Do Nutrients Counteract the Acute Cardiovascular Effects of Air Particles? The Role of Immuno-Epigenetics in Observational and Intervention Studies

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The Role of Immuno-epigenetics in Observational and Intervention Studies

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A Dissertation Submitted to the Faculty of

The Harvard T.H. Chan School of Public Health

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Science

in the Department of Environmental Health

Harvard University

Boston, Massachusetts

May 2016

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Abstract

Background: Fine particulate matter ($PM_{2.5}$) pollution is a major risk factor for cardiovascular diseases. Identifying modifiable factors and preventative strategies to mitigate the cardiovascular effect of $PM_{2.5}$ is essential to aid the development of personalized intervention.

Method: Chapter one and two were based on a crossover human intervention trial using B vitamin supplementation (2.5 mg/d folic acid, 50 mg/d vitamin B_6, and 1 mg/d vitamin B_{12}). Ten volunteer received three two-hour controlled exposure experiments to medical air or $PM_{2.5}$ ($250\mu g/m^3$), in pre-determined order. Chapter three utilized the Normative Aging Study, a longitudinal cohort study with 573 elderly men. We used electrocardiogram to measure resting heart rate (HR) and heart rate variability (HRV), hematology analyzer to determine white blood cell (WBC) counts, and Infinium HumanMethylation450 BeadChip to measure DNA methylation in peripheral CD4+ T helper (T_h) cells (pre-, post-, 24-hr post-exposure). Blood *TLR2* methylation was analyzed using pyrosequencing. Daily flavonoid and methyl nutrients intakes were assessed through the Food Frequency Questionnaire.

Results: Compared to medical air, $PM_{2.5}$ exposure was associated with 3.8 beat/min (95% CI, 0.3, 7.4; *P*=0.04) higher resting HR, 57.5% (95% CI, 2.5%, 81.5%; *P*=0.04) lower low-frequency (LF) power, and altered DNA methylation landscape, following exposure. $PM_{2.5}$ exposure was associated with 11.5% (95% CI, 0.3%, 24.0%; *P*=0.04) higher total WBC count and 12.9% (95% CI, 4.4%, 22.1%; *P*=0.005) higher lymphocyte count, at 24-hour post-exposure.

These effects of $PM_{2.5}$ were abrogated with B vitamins supplement. In the Normative Aging Study, every 10 $\mu g/m^3$ increase in 48-hour $PM_{2.5}$ moving average was associated with 7.74% (95%CI: -1.21%, 15.90%; *P*=0.09), 7.46% (95%CI: 0.99%, 13.50%; *P*=0.02), 14.18% (95%CI, 1.14%, 25.49%; *P*=0.03), and 12.94% (95%CI, -2.36%, 25.96%; *P*=0.09) reductions in root mean square of successive differences (rMSSD), standard deviation of normal-to-normal intervals (SDNN), LF power, and high-frequency (HF) power, respectively. Higher *TLR2* methylation exacerbated the rMSSD, SDNN, LF, and HF reductions associated with heightened $PM_{2.5}$ ($P_{interaction}$=0.006, 0.03, 0.05, 0.04, respectively). Every interquartile-range increase in flavonoid intake was associated with 5.09% reduction in mean *TLR2* methylation (95%CI, 0.12%, 10.06%; *P*=0.05) and counteracted the effects of $PM_{2.5}$ on LF ($P_{interaction}$=0.05).

Conclusions: Ambient $PM_{2.5}$ exposure peak has unfavorable effect on cardiac autonomic function, the immune system, and the epigenome – which, can be counteracted by B vitamins supplementation. In addition, the epigenetic regulation of TLR2-related immunity may determine vulnerability of older individuals when confronted with air pollution peaks.

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log_{10}HR indicates log_{10}-transformed heart rate; log_{10}rMSSD indicates log_{10}-transformed root mean square of the successive differences; log_{10}SDNN indicates log_{10}-transformed standard deviation of normal-to-normal intervals; log_{10}LF indicates log_{10}-transformed low-frequency power (0.04 –0.15 Hz); log_{10}HF indicates log_{10}-transformed high-frequency power (0.15–0.4 Hz); Q1, Q2, Q3, and Q4 indicate the 1st, 2nd, 3rd, and 4th quartiles.

The association of $PM_{2.5}$ with rMSSD, SDNN, LF, and HF is modified by mean *TLR2* methylation levels, as indicated by the different slopes. The four lines in each figure represent the relationship between $PM_{2.5}$ and HRV when the mean *TLR2* methylation level is at the midpoints of each quartile. If there was no effect modification, the four lines would be the same. The P_{global} for Interaction was based on a global test for effect modification by position-specific methylation. Interaction terms between each of the five *TLR2* positions and $PM_{2.5}$ were included in the model, and these five interaction terms were tested jointly for effect modification using a Wald test (global test). Results were adjusted for age; body mass index; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise, household income; the

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IQR indicates interquartile-range. Results were adjusted for age, body mass index, total caloric intake, total vitamin C intake, total fiber intake, smoking status, household income, and physical activity.

Figure 3.5 Effect of dietary vitamin B12 (N=497) and methionine (N=482) intake on mean and position-specific *Toll-like Receptor 2* (*TLR2*) methylation

IQR indicates interquartile-range. Results were adjusted for age, body mass index, total caloric intake, total vitamin C intake, total fiber intake, smoking status, household income, and physical activity.

Figure 3.6 The effect of particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) exposure on heart rate variability (HRV) at different flavonoid intake levels, Normative Aging Study, 2000-2011 (N=513)

log_{10}HR indicates log_{10}-transformed heart rate; log_{10}rMSSD indicates log_{10}-transformed root mean square of the successive differences; log_{10}SDNN indicates log_{10}-transformed standard deviation of normal-to-normal intervals; log_{10}LF indicates log_{10}-transformed low-frequency power (0.04 –0.15 Hz); log_{10}HF indicates log_{10}-transformed high-frequency power (0.15–0.4 Hz); Q1, Q2, Q3, and Q4 indicate the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} quartiles.

The association of $PM_{2.5}$ on rMSSD, SDNN, LF, and HF is modified by flavonoid intake, as indicated by the different slopes. The four lines in each figure represent the relationship between $PM_{2.5}$ and HRV when the flavonoid intake at the midpoints of each quartile. If there was no

effect modification, the four lines would be the same. Results were adjusted for age; body mass index; total caloric intake; total vitamin C intake; total fiber intake; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise; household income; the use of calcium channel blocker, β-blocker, and angiotensin-converting enzyme inhibitor; room temperature; outdoor apparent temperature; season; weekday; and visit date.

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Acknowledgements

The dissertation would not have been possible without the help of so many people in so many ways. I am extremely fortunate to have Dr. Andrea Baccarelli as my mentor, whose mentoring style is a great balance of scientific expertise, advice, and support, while also encouraging my own independence. Andrea encourages me to be creative and confident and cheers for every small achievement that I've made. He not only dedicated to my career development as an environmental epidemiologist, but also cares for my wellbeing and my family. Thank you, Andrea, for always believing in your students. To me, you are more than the dissertation advisor – you are my mentor, role model, and greatest inspiration.

I am grateful to have the guidance and support from my wonderful committee members, Dr. Diane Gold, Dr. Brent Coull, and Dr. Liming Liang.

Thank you, Diane, for your tremendous support, encouragement, and endless words of wisdom. While brilliant and successful, you are always so patient and approachable. Thank you for always being there for me when I needed help. I deeply appreciate your trust and care, and will for sure miss our productive and enjoyable Tuesday morning meetings.

Dr. Brent Coull is truly the best statistical advisor that a student can hope for. He is so knowledgeable and generous in sharing his expertise. Thank you, Brent. Your concrete feedback to my projects directed me to the correct path towards finding a solution to any problem. I have always valued any opinion or advice you had to offer and appreciate the genius behind them.

Dr. Liming Liang, I appreciate so much for all the advice, knowledge, and resources you have provided. Thank you for always making time for my questions, and for always being willing to

help. Your creative and constructive suggestions have made tremendous contributions to my projects.

I would like to thank all my professors, friends and colleagues. I am honored and humbled to be surrounded with wonderful people, who helped me so much throughout these years and made the four years at Harvard a lovely memory.

This dissertation is dedicated to my family – Lili, Juncheng, Guan, Ryan, and Erin. You are the source of my happiness and the purpose of my life. I am grateful to have kind, trusting, and loving parents, Juncheng and Lili, without whom I wouldn't have a chance to pursue the life I wanted.

Thank you, Guan, for being there with me through good times and tough times. Your passion and enthusiasm in science is the greatest inspiration that motivates me. You are the best demonstration of hard-working spirit and steadfast dedication to a career that you love. Thank you for making me a better person, and for the unweaving support. Without you, nothing would be possible.

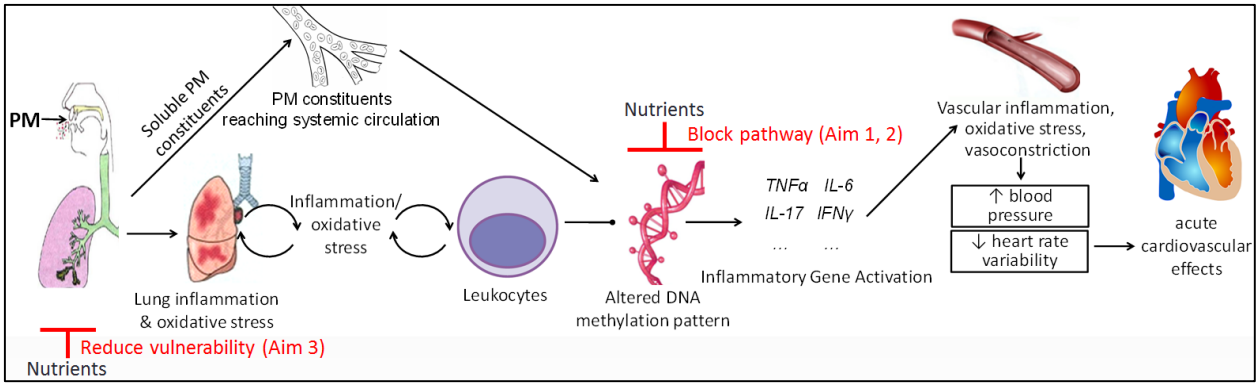
Finally I want to thank my dearest children, Ryan and Erin. There is nothing like your laughter and innocent faces to bring out the strongest side of me. It was all your magic that keeps me focused, motivated, and happy. You are the reason why I want to devote my life promoting public health. I hope all that we do, will make positive impacts on the world that you live, and many generations to come.

Introduction

1. Air pollution: impact on CVD and need for preventive strategies

Particulate Matter (PM) has been estimated to contribute to 3.7 million deaths worldwide each year.[11] According to the American Heart Association (AHA), PM exposure is a modifiable primary contributor to cardiovascular morbidity and mortality, often resulting from acute cardiovascular effects after short-term exposure peaks.[2] $PM_{2.5}$ is especially deleterious because it penetrates into the alveoli and may act as a stimulus to trigger local cytokine production and systemic inflammation.[12, 13] The major sources of $PM_{2.5}$ pollution are combustion activities (motor vehicles, power generation, and industrial processes), biomass burning, and other human activities such as heating and cooking.[2] Because prevention of exposure to PM is particularly challenging—more than 80% of people in the United States (US) receive some exposure—it is essential to 1). determine molecular intervention targets underlying the cardiovascular effects of PM (*Aim 1 and 2*) (Fig. 1); 2). identify modifiable factors that contribute to individual susceptibility to cardiac effects to aid the development of effective interventions (*Aim 3*) (Fig. 0.1).

Figure 0.1: Proposed Conceptual Model



2. Reduced HRV and elevated BP: ideal early markers of PM-induced cardiovascular system impairment

Impaired autonomic modulation of the rhythmic activity of the sinus node, as reflected in reduced HRV, represents a pathophysiologic mechanism by which air pollution may lead to cardiac mortality, especially among older individuals.[14, 15] The negative association between short-term $PM_{2.5}$ exposure and HRV has been repeatedly observed in general population,[16] cardiac and hypertensive patients,[17] asthmatic adults,[18] young adults,[19] and elderly adults.[20, 21] Notably, HRV shows wide inter-individual variability, as well as highly variable responses to PM exposure.[7] Therefore, HRV is an ideal early surrogate marker of cardiovascular autonomic dysfunction for identifying underlying factors that may modify susceptibility to cardiovascular effects of PM exposure.

Further, effect of short-term exposure to PM on rapidly increased BP has been demonstrated in observational[4, 5] and controlled human exposure studies to concentrated ambient particles (CAPs).[22-25] In particular, the adverse effect of short-term ambient PM on BP has been observed in general population,[26] healthy adults,[24, 27] older adults,[28] cardiac disease patients,[4, 29] and patients with lung disease.[30, 31] Our previous experiments of controlled human exposure to CAPs have reproducibly shown rapid adverse effects on BP as early as two hours after exposure to PM.[22-25, 32] Taken together, elevated BP in response to air pollution peaks have been indicated as one of the primary intermediates leading to acute air pollution-related CVD events.[2, 5]

3. Immuno-epigenetics: the dynamic epigenetic regulation of the immune system

Growing evidence indicates that systemic inflammation not only is an essential mechanistic pathway linking adverse cardiovascular events following PM exposure,[2, 33] but also exacerbates disturbances on autonomic modulation following PM exposure.[8] Thus, the regulation of the immune system is critical to limiting immune-mediated cardiovascular pathology.[10] DNA

methylation, the most studied epigenetic process, is shown to be a key mechanism modulating the activity of inflammatory genes post PM exposure.[8, 9, 34-37] In turn, inflammation and oxidative stress can influence both global and gene-specific methylation. Taken together, these findings indicate the unique roles of this dynamic crosstalk between the epigenome and the immune system, and potential opportunity to target the epigenome for avoiding immune-mediated adverse cardiovascular effects.

3.1. Hypomethylation of inflammatory genes in T-helper (T_h) cells: novel mechanism underlying the cardiovascular effects of air pollution Observational studies has repeatedly shown that PM exposure can induce hypomethylation in blood leukocyte DNA, particularly of inflammatory genes.[8, 34-37] Based on these findings, blood leukocyte DNA hypomethylation has been indicated as a novel mechanism activating the expression of inflammatory genes and potentially linking PM inhalation to CVD effects.[9] T_h cells have been shown as a central blood cell type which responds to PM exposure peaks with rapid DNA demethylation and mediate acute cardiovascular effects. Hypomethylation and expression of inflammatory cytokines in T_h cells were observed as early as 2 hours after an antigen challenge.[38] Inhalation of diesel PM has been specifically linked to inflammatory responses and interferon-gamma hypomethylation in circulating T_h cells from exposed rats.[37] RAG-$1^{-/-}$ mice, which lack T cells, have markedly blunted hypertensive responses to angiotensin II and salt challenges, and researchers were able to reinstate the normal responses by restoring T cells to these animals.[39] Subsequent studies have demonstrated that cytokines released by T_h cells contributed to the observed phenomenon.[40, 41] These evidences have shown the critical roles for T_h-cell mediated cytokines production in regulating the adaptive immunity and immune cell proliferative responses.[42] In addition, recent

evidences suggested that T_h cells are crucial players involved in vasoconstriction and hypertension. Mice lacking interleukin-17 (IL-$17^{-/-}$ mice), a Th_{17} cytokine, exhibit lower BP increases after angiotensin infusion, as well as reduced effects on endothelium-dependent vasodilatation.[43] This growing body of work strongly suggests mechanistic roles for cytokines released from T_h cells in the production of rapid adverse BP and endothelial responses.[39, 40, 42-44]

3.2. Hypermethylation of immune-regulatory gene TLR2: epigenetic susceptibility to PM-induced cardiovascular effects Molecules involved in immunoresponses are attractive as potential modulators of cardiovascular pathology following PM exposure. Toll-like receptors (TLRs), a group of receptors abundantly expressed on leukocytes, have emerged as crucial first-responders linking innate and adaptive immunity after environmental challenge.[45] TLR2, in particular, is a unique TLR family member that not only assists the clearance of bacterial components contained in PM via pathogen recognition,[46, 47] but also modulates the expansion and behavior of regulatory T cells (T_{reg})—the dominant circulating regulator of immunosuppression.[48, 49] Netea et al. reported a 50% decrease in the number of CD4+CD25+ T_{reg} cells accompanied by an impaired release of immunosuppressive cytokines in *TLR2* null mice compared with wild-type mice, demonstrating the role of TLR2 in immunoregulation.[49] These findings support our hypothesis that high *TLR2* methylation, an epigenetic process associated with suppressed *TLR2* expression, conveys susceptibility to PM exposure-induced HRV reductions by decreasing immunoregulation. *TLR2*-related immunity is controlled through epigenetic mechanisms: increased methylation in the *TLR2* promoter region is usually associated with *TLR2* silencing, while decreased methylation permits *TLR2* expression.[50] Therefore, maintaining a proper methylation level of the *TLR2* gene is critical to ensure protective immunity,

while dysregulated *TLR2* level might contributes to "epigenetic susceptibility" for immune-mediated PM-induced cardiovascular responses.

4. Flavonoid and methyl-donors: Unique opportunities for human translation of animal findings

DNA methylation is reversible, thereby providing unique opportunities for modulation. This feature is critical, particularly for pathologies related to non-preventable exposures, as it can offer potential interventions to ameliorate exposure-related disease. Nutrients such as flavonoids and methyl nutrients can modify gene-specific methylation in opposite directions.[51-54] Flavonoids, the most common group of polyphenolic compounds in the human diet, can lower DNA methylation and inhibit methylation-induced gene silencing.[52, 53] Specifically, flavonoids inhibit the activity of DNA methyltransferase, reverse DNA hypermethylation and can reactivate silenced genes.[52, 53] Lee and coauthors demonstrated a negative dose-response relationship between flavonoid concentration and enzyme-mediated DNA methylation.[55] Methyl nutrients, on the other hand, may increase methylation by providing methyl donors.[54] Thus, determining how such nutrients alter methylation states can provide insight that may be used to counteract exposure effects.

The observation that PM induces DNA hypomethylation opens up an unprecedented opportunity for human translation of epigenetic modulation that has been successfully used in animal models.[56] DNA methylation is dependent on a biochemical cycle relying on dietary methyl donors, including folic acid, vitamin B_6 and B_{12}, and methionine.[57, 58] The classic Agouti A^{vy} mice experiments have shown that dietary methyl donors supplement during gestation[57, 59] or in adult life[60-62] can increase DNA methylation. Dolinoy et al.[63] showed that methyl donors can

avert Bisphenol A-induced hypomethylation, suggested the potential use of methyl donors to protect the adverse effect of environmental toxins. In epidemiological studies, methyl donor intake/plasma methyl donor is positively associated with global blood DNA methylation status in adults.[64-66] Further, cultured human T cells undergo gene demethylation when challenged with low folate and methionine culturing environment.[67] Taken together, these findings suggested the critical impact of methyl donor on DNA methylation in adults, and highlighted that DNA methylation in T cells is specifically dependent on methyl donor availability.

No study has yet investigated whether DNA methylation and nutrient intake may modulate the cardiovascular effects of air pollution exposure. In Aim 1 and 2, we aim to address a fundamental pharmacologic and mechanistic question: does exposure have epigenetic effects with downstream subclinical/clinical cardiovascular consequences, and can adverse effects be safely reduced pharmacologically? In Aim 3, we hypothesize that programming of *TLR2* inactivation by increased methylation contributes to "epigenetic susceptibility" for immune-mediated PM-induced cardiovascular responses, and dietary nutrients intake could affect the epigenetic programming of *TLR2*.

INNOVATION: To the best of our knowledge, our study will be the first to: **1.** test the effects of a human intervention and translate a wealth of animal data showing that methyl-donors can be used to modulate epigenetic states and avert environmental effects (*Aim 1*); **2.** utilize controlled human exposure to PM, an approach that can reproduce ambient peaks of PM exposure (*Aim 1 and 2*); **3.** examine DNA methylation and expression in isolated T_h, a key cell type mediating hypertensive responses (*Aim 2*); **4.** test the role of the interplay between nutrient intake and DNA methylation on pollution-induced cardiovascular responses (*Aim 3*).

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Chapter 1

A Human Intervention Trial: Effects of Fine Concentrated Ambient Particles on Cardiac Autonomic Dysfunction and Inflammation Are Mitigated by B vitamin supplement

Background: Ambient fine particulate matter ($PM_{2.5}$) pollution is a trigger of acute cardiovascular events. Individual-level preventions have been proposed to complement regulation in reducing the global burden of $PM_{2.5}$–related cardiovascular diseases. We investigated whether B vitamins mitigate the acute effects of $PM_{2.5}$ on cardiac autonomic dysfunction and inflammation.

Methods: In a crossover human intervention trial, we administrated placebo and then four-week B vitamin supplement (2.5 mg/d folic acid, 50 mg/d vitamin B_6, and 1 mg/d vitamin B_{12}) to ten healthy volunteers. Each volunteer received three two-hour controlled exposure experiments to medical air or $PM_{2.5}$ (250μg/m^3), in pre-determined order. We used electrocardiogram to measure resting heart rate (HR) and heart rate variability (HRV), and hematology analyzer to determine white blood cell (WBC) counts (pre-, post-, 24-hr post-exposure).

Results: Compared to medical air, $PM_{2.5}$ exposure was associated with 3.8 beat/min (95% CI, 0.3, 7.4; *P*=0.04) higher resting HR and 57.5% (95% CI, 2.5%, 81.5%; *P*=0.04) lower low-frequency power following exposure. $PM_{2.5}$ exposure was associated with 11.5% (95% CI, 0.3%, 24.0%; *P*=0.04) higher total WBC count and 12.9% (95% CI, 4.4%, 22.1%; *P*=0.005) higher lymphocyte count, at 24-hour post-exposure. These $PM_{2.5}$ effects were mitigated by B vitamin supplement – which attenuated the effect of $PM_{2.5}$ on HR by 90% (*P*=0.003), low-frequency power by 96% (*P*=0.01), total WBC count by 139% (*P*=0.006), and lymphocyte count by 106% (*P*=0.02).

Conclusions: In healthy adults, two-hour $PM_{2.5}$ exposure substantially increases HR, reduces HRV, and increases WBC. These effects were curbed by four-week B vitamin supplement.

INTRODUCTION

Ambient fine particulate matter ($PM_{2.5}$) pollution accounts for 3.7 million premature deaths per year worldwide, predominantly due to its acute effects on the cardiovascular system.[1] A recent meta-analysis concluded that PM pollution is the most frequent trigger for myocardial infarction at the population level.[2] Even at levels below the current National Air Quality Standards, associations of $PM_{2.5}$ exposure with increased cardiovascular risk have been found in sensitive individuals.[3,4] Moreover, many urban areas outside of North America continue to have elevated levels of $PM_{2.5}$ pollution.[1,5] Definition of options for individual-level prevention that may complement regulation to reduce the global burden of cardiovascular disease (CVD) due to ambient $PM_{2.5}$ pollution represents a critical gap in current knowledge.[4]

Reduced heart rate variability (HRV), reflecting perturbation of autonomic function,[6,7] is a sensitive marker that can change rapidly in response to $PM_{2.5}$ exposure.[3] It represents a primary pathophysiologic intermediate that might leads to PM-related adverse cardiovascular events.[4] In the Normative Aging Study, we found that the associations of short-term $PM_{2.5}$ exposure with reduced HRV was limited to subjects with lower intakes of vitamin B_6 or B_{12} and was abrogated in those with higher intakes.[6] These findings suggest that B vitamins provide protection against the effect of $PM_{2.5}$ on the autonomic nervous system.

Higher B vitamins (folic acid, vitamins B_6, and B_{12}) intake is associated with decreased CVD risk in epidemiologic studies.[8,9] However, to date, the results from randomized clinical trials do not support the benefit of B vitamin supplementation for CVD prevention.[10-15] Recent studies suggest that B vitamins might be effective to minimize health effects of environmental stressors

because of its anti-inflammatory and antioxidant properties. [6,16] In animal models, B vitamin supplementation has been successfully used to curb the oxidative stress, inflammation, and metabolic phenotype change due to environmental stressors.[17,18] However, no clinical trial has yet investigated whether B vitamin supplementation impacts the biologic response to ambient air pollution exposure.

Our study is the first human trial to evaluate whether B vitamin supplementation (folic acid, vitamin B_6 and B_{12}) can curb the acute autonomic effects of $PM_{2.5}$. We conducted a single-blind crossover intervention trial with controlled human exposure experiments to fine concentrated ambient particles (fine CAP, or $PM_{2.5}$), on ten healthy adults. Because of the central role of inflammation in modulating the cardiovascular effects of $PM_{2.5}$, we also investigated the $PM_{2.5}$ effect on total and differential white blood cell (WBC) counts, and the potential for B vitamins to counteract these effects.

METHODS

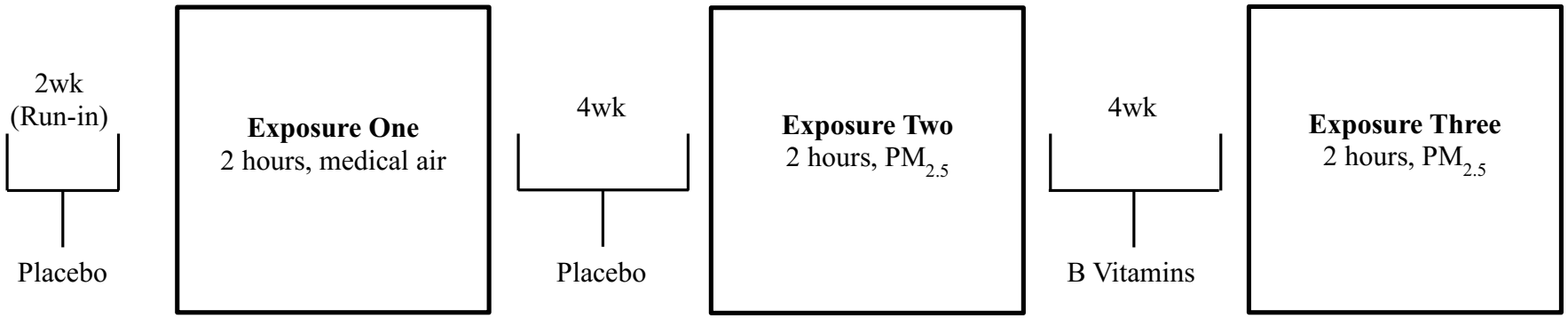
Study population

We recruited ten healthy, 18–60 year old, non-smoking volunteers who are not on any form of B vitamin supplement, from the University of Toronto campus and surrounding area. The trial was approved by all participating institutional review board and registered. All volunteers provided written informed consent before enrolling. The conduct of the trial was monitored by an independent data and safety monitoring committee.

Study design

We conducted a single-blind crossover intervention trial (Figure 1.1) with controlled exposure experiments (July 2013 to February 2014). A two-hour blank exposure experiment (exposure one, particle-free medical air) was included to provide baseline data. All volunteers then received placebo for four weeks preceding the two-hour exposure experiment to concentrated ambient $PM_{2.5}$ (exposure two, 250 $\mu g/m^3$). After exposure two, we administered B vitamin supplement for four weeks before the next two-hour exposure experiment to $PM_{2.5}$ (exposure three, 250 $\mu g/m^3$). We used a four-week washout period between exposure experiments because it was judged to be sufficient to diminish the carryover effect of $PM_{2.5}$ exposure,[19,20] while minimizing the impact of seasonality and temporal trend on the source and composition of the concentrated ambient $PM_{2.5}$. In order to ensure comparable conditions across all controlled exposure experiments to $PM_{2.5}$, the present study could not randomize on the treatment (placebo *vs* B vitamins) sequence because vitamin B_{12} has a biological half-life longer than four months.[21] Study volunteers are blinded to exposure and treatment allocation. Based on our symptom survey, none of the volunteer was able to discern the exposure type for any exposure experiment.

Figure 1.1 Study design: A cross-over intervention trial with controlled exposure experiments



Exposure facility

Harvard fine and coarse particle concentrators with a dilution control system delivered target-concentration $PM_{2.5}$,[22] and the medical air exposures were generated as previously described.[23] The concentrated ambient $PM_{2.5}$ air stream was delivered directly to the volunteer who is seated inside a 4.9 m^3 (1.1 x 1.9 x 2.0 m) Lexan enclosure, at rest and breathing freely via an "oxygen type" facemask on his/her nose and mouth. During each exposure experiment to $PM_{2.5}$, particles were collected on polycarbonate membrane filters and we monitored gravimetric determination of $PM_{2.5}$ exposure mass concentration ($\mu g/m^3$).

Folic acid, vitamin B_6, and vitamin B_{12} supplement

We administered one placebo or B vitamin supplement (2.5 mg folic acid, 50 mg vitamin B_6, and 1 mg vitamin B_{12}) daily. Preparation and packaging of the placebo and B vitamin supplement was done by an external lab (Jamieson Laboratory, Toronto, Canada) to ensure blinding. The coding used on the label was blinded to the volunteers. We monitored volunteers' plasma folic acid, vitamin B_6 and B_{12} levels prior to each exposure experiment. Self-administered validated semi-quantitative Food Frequency Questionnaire was used to assess dietary B vitamin intake at the first and the last visit, to rule out potential impact from diet.

Heat rate, heart rate variability, and WBC measurement

We measured supine resting heart rate (HR) and HRV using high-resolution (1 KHz sample rate) digital 12-lead holter electrocardiogram monitors (H12+ recorder, Mortara Instruments, Milwaukee, WI). We obtained HRV measurements before (pre-exposure) and after (immediately post-exposure and 24-hour post-exposure) each exposure experiment. We extracted ten-minute

resting supine HRV readings on time domain outcomes (standard deviation of NN intervals (SDNN), square root of the mean squared differences of successive NN intervals (rMSSD), proportion of successive NN intervals with differences > 50 msec (pNN50)) and frequency domain outcomes (low-frequency (LF) power, high-frequency (HF) power, and their ratio (LF/HF)).[24] We discarded the first three minutes and the last two minutes during the ten-minute recording and analyzed the remaining five-minute electrocardiogram data using standardized techniques.[25] SDNN represents the total variability. PNN50, rMSSD, and HF are sensitive to high-frequency heart rate fluctuations and are considered as measures of cardiac vagal tone modulation, while LF power is linked to the activity of both sympathetic and parasympathetic nervous system.

Blood samples (pre-, post-, and 24 hour post-exposure) were obtained in EDTA vacutainer tubes and stored at 4 °C, and subsequently processed in a local laboratory within two hours for total and differential WBC counts using the Technicon H-1 automated hematology analyzer (Technicon Instruments Corp, Terrytown, NY, USA).

Statistical methods

We conducted graphical explorations and $\log_{10}$-transformed HRV measures and WBC counts to improve normality and stabilize the variance.[19] We examined the linear relationships between HR/HRV/WBC and all independent variables and covariates, and observed no deviation from linearity. For the ease of interpretation, we scaled the effect estimates to the percent changes in HRV and WBC in all models.

We used linear mixed-effects models with a robust/sandwich estimator for the variance (**Model 1**) to account for within-subject correlation in the outcome measures. Random intercepts were assigned to each subject. In all models, we adjusted for covariates with potential influences on HR, HRV, and WBC, selected based on prior knowledge and the existing literature, i.e., season (fall/winter/spring/summer), chamber temperature, and relative humidity.[15,16]

$$Y_{ij} = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{1ij} * X_{2ij} + \beta_3 X_{3ij} + \ldots + \beta_p X_{pij} + b_i + \varepsilon_{ij} \qquad \text{(Model 1)}$$

In the above model, Y_{ij} was the change in HR, HRV, or WBC (i.e., ΔHR= post-exposure HR – pre-exposure HR) for participant i at exposure occasion j. β_0 was the overall intercept, and b_i was the separate random intercept for subject i with, $b_i \sim N(0, \Theta)$, $\varepsilon_{ij} \sim N(0, \sigma^2)$. X_{1ij} was a binary variable indicating exposure to $PM_{2.5}$ or medical air. X_{2ij} was a binary variable indicating placebo or B vitamin supplement. X_{3ij}–X_{pij} were the covariates, for participant i at measurement j. The main effect of B vitamin supplement was not included in the model, given volunteers did not receive any medical air exposure while on B vitamin supplement. β_1 represents the effect of $PM_{2.5}$ exposure (without B vitamin supplement) and β_2 represents the effect modification by B vitamin supplement. A two tailed value of $P \leq 0.05$ was considered statistically significant. Analyses were performed using SAS 9.4 (SAS Institute, Cary NC).

RESULTS

Study population characteristics and exposure levels

All volunteers completed three controlled exposure experiments. Four volunteers were white, three were Asian, and three were other races. Six volunteers were female and three had a BMI ≥25 (Table 1.1). The baseline mean resting HR was 58.9 beat/min (range: 43.0 to 74.0 beat/min). The baseline resting HR did not vary substantially by age, gender, race, or being overweight (Table 1.1).

The target $PM_{2.5}$ concentrations were controlled by design; however, there was some variation in the actual $PM_{2.5}$ concentration (Table 1.2). Among all controlled exposures to $PM_{2.5}$, $PM_{2.5}$ concentration varied from 100.6 $\mu g/m^3$ to 287.5 $\mu g/m^3$, with a median of 234.0 $\mu g/m^3$. Previous studies using the same exposure facility reported minimal concentration of $PM_{2.5}$ in medical air (median=0.0 $\mu g/m^3$, interquartile range=2.40 $\mu g/m^3$).[19] There was no significant difference in $PM_{2.5}$ concentration between exposure 2 and 3 (P=0.38) (Table 1.2).

Plasma concentrations of B vitamins

Four-week B vitamin supplement significantly increased the plasma concentration of folic acid, vitamins B_6 and B_{12} (P=0.02, P=0.004, P=0.01; respectively), while placebo did not alter the plasma folic acid, vitamins B_6 and B_{12} levels (P=0.82, P=0.75, P=0.42; respectively) (Table 1.3).

Effect of $PM_{2.5}$ on HR, HRV, and WBC without B vitamin supplement

In the absence of B vitamin supplement, $PM_{2.5}$ exposure increased HR and lowered HRV after exposure (Table 1.4). Compared to medical air, two-hour $PM_{2.5}$ exposure was associated with 3.8

Table 1.1 Baseline characteristics of the study participants (N=10)

Characteristics	N (%)	Resting Heart Rate Mean (SD)
Age (years)		
19-29	7 (70)	62.3 (8.1)
30-49	3 (30)	51.0 (7.0)
Gender		
Female	6 (60)	61.0 (8.9)
Male	4 (40)	55.8 (10.0)
Race		
White	4 (40)	52.0 (6.2)
Asian	3 (30)	64.3 (8.5)
Other	3 (30)	62.7 (9.5)
BMI (kg/m^2)		
<25	7 (70)	62.1 (8.2)
≥25	3 (30)	51.3 (7.6)

SD indicates standard deviation; BMI indicates body mass index.

Table 1.2 Total exposure mass concentration of fine particles ($PM_{2.5}$) across three exposure experiments

Exposure*	Mean (SD)	Median (IQR)	Maximum	Minimum	*P* for T test
Exposure 1 (Medical Air + Placebo) ($\mu g/m^3$)	--	--	--	--	--
Exposure 2 ($PM_{2.5}$ + Placebo) ($\mu g/m^3$)	217.1 (50.6)	219.1 (33.1)	100.6	287.5	0.38
Exposure 3 ($PM_{2.5}$ + B vitamins) ($\mu g/m^3$)	233.8 (31.2)	237.2 (48.7)	184.6	286.2	

SD indicates standard deviation; IQR indicates inter-quartile range.

*Integrated gravimetric (filter sample) 2-hour exposure concentrations sampled from $PM_{2.5}$/medical air airstream inlet to human exposure chamber.

Table 1.3 Plasma concentration of folic acid, vitamin B_6 and B_{12}, prior to each exposure experiment

	Mean (SD)	Median (IQR)	Minimum	Maximum	*P**
	Prior to Exposure 1 (Medical Air + Placebo)				
Folic acid (nmoles/L)	37 (9)	35 (14)	28	56	--
B_6 (nmoles/L)	42 (11)	41 (1)	25	57	--
B_{12} (pmoles/L)	305 (107)	292 (72)	173	486	--
	Prior to Exposure 2 ($PM_{2.5}$ + Placebo)				
Folic acid (nmoles/L)	39 (13)	39 (24)	22	56	0.82
B_6 (nmoles/L)	87 (142)	37 (18)	19	482	0.75
B_{12} (pmoles/L)	302 (99)	262 (214)	197	441	0.42
	Prior to Exposure 3 ($PM_{2.5}$ + B vitamins)				
Folic acid (nmoles/L)	124 (186)	56 (13)	27	638	0.02
B_6 (nmoles/L)	393 (224)	428 (321)	23	708	0.004
B_{12} (pmoles/L)	507 (166)	511 (85)	176	794	0.01

SD indicates standard deviation; IQR indicates inter-quartile range.
*P value from the Wilcoxon signed-rank test, the measurement took prior to Exposure 1 was used as the reference group.

Table 1.4 Immediate change of post- *vs* pre-exposure heart rate (HR) and heart rate variability (HRV) associated with two-hour exposure to fine particles ($PM_{2.5}$), and the attenuated $PM_{2.5}$ effect due to B vitamin supplement (N=10)

	Effect of $PM_{2.5}$ without B vitamin supplement			Effect of $PM_{2.5}$ with B vitamin supplement			
Outcome	Estimate	*P*	95% CI	Estimate	*P*	95% CI	*P* for interaction
ΔHR (beat/min)	3.84	**0.04**	0.29 to 7.40	-1.93	0.14	-4.59 to 0.73	**0.003**
	% Change*	*P*	95% CI	% Change*	*P*	95% CI	
Time Domain							
ΔSDNN	-33.56	0.06	-56.75 to 2.05	-14.55	0.47	-45.62 to 34.28	0.16
ΔrMSSD	-14.80	0.25	-35.92 to 13.27	-0.39	0.97	-23.05 to 28.94	0.26
ΔPNN50	-29.92	0.15	-57.37 to 15.22	-6.78	0.79	-46.42 to 62.18	0.15
Frequency Domain							
ΔLF	-57.49	**0.04**	-81.47 to -2.50	-5.79	0.89	-61.03 to 127.76	**0.01**
ΔHF	-34.32	0.20	-66.23 to 27.72	-6.47	0.81	-48.18 to 68.82	0.19
ΔLF/HF	-35.93	0.09	-61.81 to 7.50	-1.56	0.94	-37.49 to 55.01	0.06

HR indicates heart rate; SDNN indicates the standard deviation of normal-to-normal (NN) intervals; rMSSD indicates the root mean square of successive differences; PNN50 indicates percentage of differences between adjacent NN intervals that are greater than 50 milliseconds; LF indicates low-frequency power (0.04 –0.15 Hz); HF indicates high-frequency power (0.15–0.4 Hz).
* Represents the % change in post-exposure HRV/pre-exposure HRV ratio associated with $PM_{2.5}$ exposure, compared to medical air. Results were adjusted for chamber humidity, chamber temperature, and season (Spring/Summer/Fall/Winter).

beat/min (95% CI, 0.3 beat/min, 7.4 beat/min; *P*=0.04) higher resting HR. $PM_{2.5}$ exposure was associated with 33.6% (95% CI, -2.1%, 56.8%; *P*=0.06), 57.5% (95% CI, 2.5%, 81.5%; *P*=0.04), and 35.9% (95% CI, -7.5%, 61.8%; *P*=0.09) lower SDNN, LF, and LF/HF ratio compared to medical air, respectively (Table 1.4). At 24-hour post-exposure, we did not observe any significant effect of $PM_{2.5}$ exposure on HR or HRV (Table 1.5).

Exposure to $PM_{2.5}$ increased total and differential WBC counts, compared to medical air (Table 1.6). Immediately after exposure, $PM_{2.5}$ was associated with 9.9% (95% CI, -0.8%, 21.8%; *P*=0.07) and 10.0% (95% CI, -1.7%, 23.2%; *P*=0.09) higher numbers of total WBC and lymphocyte (Table 1.6). At 24-hour post-exposure, $PM_{2.5}$ exposure was associated with 11.5% (95% CI, 0.3%, 24.0%; *P*=0.04) and 12.9% (95% CI, 4.4%, 22.1%; *P*=0.005) higher numbers of total WBC, neutrophil, lymphocyte, and monocyte, respectively (Table 1.6).

B vitamin supplement attenuated the effects of $PM_{2.5}$

After four-weeks of B vitamin supplementation, the associations were significantly attenuated. For example, the association of $PM_{2.5}$ with post-exposure HR (*P* for interaction=0.003), HRV (*P* for interaction=0.01 for LF), and total WBC count (*P* for interaction=0.008). The effect of $PM_{2.5}$ on HR was diminished and was no longer significant with B vitamin supplement (-1.9 beat/min, 95% CI, -4.6 beat/min, 0.7 beat/min; *P*=0.14) (Table 1.4). Likewise, B vitamin supplementation resulted in a reduction in the effect size of $PM_{2.5}$ by 90% for LF, and 96% for LF/HF ratio (Table 4). With B vitamin supplement, exposure to two-hour $PM_{2.5}$ was associated with 5.8% (95% CI, -127.8%, 61.0%; *P*=0.89; *P* for interaction=0.01) and 1.6% (95% CI, -55.0%, 37.5%; *P*=0.94; *P* for interaction=0.06) lower LF and LF/HF ratio, respectively (Table 1.4). In addition, although

Table 1.5 Change of 24-hour post- *vs* pre-exposure heart rate (HR) and heart rate variability (HRV) associated with two-hour exposure to fine particles ($PM_{2.5}$), and the attenuated $PM_{2.5}$ effect due to B vitamin supplement (N=10)

	Effect of $PM_{2.5}$ without B vitamin supplement			Effect of $PM_{2.5}$ with B vitamin supplement			
Outcome	Estimate	*P*	95% CI	Estimate	*P*	95% CI	*P* for interaction
ΔHR (beat/min)	1.71	0.37	-2.29 to 5.71	-0.35	0.89	-5.70 to 5.01	**0.003**
	% Change*	*P*	95% CI	% Change*	*P*	95% CI	
Time Domain							
ΔSDNN	-0.43	0.98	-26.25 to 34.45	10.95	0.46	-17.59 to 49.39	0.54
ΔrMSSD	-21.87	0.16	-45.40 to 11.81	10.23	0.60	-26.19 to 64.61	**0.01**
ΔPNN50	-26.28	0.43	-67.73 to 68.40	38.88	0.42	-41.70 to 230.88	**0.03**
Frequency Domain							
ΔLF	8.37	0.62	-23.67 to 53.87	58.34	0.08	-5.54 to 165.40	0.13
ΔHF	-27.54	0.38	-66.45 to 56.51	41.97	0.39	-39.82 to 234.88	0.06
ΔLF/HF	26.70	0.57	-48.15 to 209.61	19.77	0.59	-41.33 to 144.54	0.90

HR indicates heart rate; SDNN indicates the standard deviation of normal-to-normal (NN) intervals; rMSSD indicates the root mean square of successive differences; PNN(10/20/50) indicates percentage of differences between adjacent NN intervals that are greater than (10/20/50) milliseconds; LF indicates low-frequency power (0.04 –0.15 Hz); HF indicates high-frequency power (0.15–0.4 Hz).

* Represents the % change in 24-hour post-exposure HRV/pre-exposure HRV ratio associated with $PM_{2.5}$ exposure, compared to medical air. Results were adjusted for chamber humidity, chamber temperature, and season (Spring/Summer/Fall/Winter).

Table 1.6 Change of white blood cell (WBC) counts and distribution associated with two-hour exposure to fine particles ($PM_{2.5}$), and the attenuated $PM_{2.5}$ effect due to B vitamins treatment (N=10)

	Effect of $PM_{2.5}$ without B vitamin supplement			Effect of $PM_{2.5}$ with B vitamin supplement			
	% Change*	*P*	95% CI	% Change*	*P*	95% CI	*P* for interaction
			Immediate post-pre change in WBC				
ΔWBC	9.89	0.07	-0.82 to 21.75	-1.68	0.67	-9.57 to 6.90	**0.008**
ΔNeutrophil	8.93	0.28	-7.47 to 28.22	-3.07	0.68	-17.41 to 13.77	0.06
ΔLymphocyte	10.02	0.09	-1.70 to 23.16	2.36	0.62	-7.37 to 13.11	0.09
ΔMonocyte	6.53	0.62	-18.81 to 39.77	1.63	0.86	-16.59 to 23.85	0.63
			24-hour post-pre change in WBC				
ΔWBC	11.54	**0.04**	0.34 to 23.99	-4.48	0.43	-15.52 to 7.99	**0.006**
ΔNeutrophil	12.94	0.15	-4.91 to 34.13	-8.40	0.32	-23.62 to 9.86	**0.01**
ΔLymphocyte	12.91	**0.005**	4.41 to 22.10	-0.82	0.87	-11.18 to 10.73	**0.02**
ΔMonocyte	5.23	0.36	-6.41 to 18.33	7.48	0.31	-7.34 to 24.67	0.61

* Represents the % change in post-exposure cell count/pre-exposure cell count ratio associated with $PM_{2.5}$ exposure, compared to medical air. Results were adjusted for chamber humidity, chamber temperature, and season (Spring/Summer/Fall/Winter).

non-significant, B vitamin supplement attenuated the $PM_{2.5}$ effect by 57% on SDNN, 97% on rMSSD, 77% on PNN50, and 81% on HF (Table 1.4).

The effect attenuation by B vitamins on the $PM_{2.5}$-HR or $PM_{2.5}$-HRV relationship (*P* for interaction=0.003, 0.01, 0.03 for HR, rMSSD, and PNN50, respectively) remained significant, at 24-hour post-exposure (Table 1.5).

The associations of $PM_{2.5}$ with post-exposure total and differential WBC counts were weakened by B vitamin supplement (Table 1.6). Compared to medical air, effects of $PM_{2.5}$ on WBCs were diminished to non-significant with B vitamin supplement: two-hour $PM_{2.5}$ exposure was associated with a -1.7% (95% CI, -9.6%, 6.9%; *P*=0.67; *P* for interaction=0.008), -3.1% (95% CI, -17.4%, 13.8%; *P*=0.68; *P* for interaction=0.06), and 2.4% (95% CI, -7.4%, 13.1%; *P*=0.62; *P* for interaction=0.09) change in total WBC, neutrophil, and lymphocyte, respectively (Table 1.4). In summary, B vitamin supplement reduced the $PM_{2.5}$ effect by 117%, 134%, 76%, and 75% on total WBC, neutrophil, lymphocyte, and monocyte, respectively (Table 1.6). B vitamin supplement non-significantly attenuated the association of $PM_{2.5}$ with monocyte (Table 1.6).

Likewise, at 24-hour post-exposure, B vitamin supplement attenuated the $PM_{2.5}$ effects on total and differential WBC counts (*P* for interaction=0.006, 0.01, 0.02, 0.61 for total WBC, neutrophil, lymphocyte, and monocyte) (Table 1.6). B vitamin supplement significantly reduced the $PM_{2.5}$ effect by 139% on total WBC, 167% on neutrophil, and 106% on lymphocyte (Table 1.6).

Sensitivity analysis

We dealt with potential influence by season via adjusting for spring/summer/fall/winter in all models. In sensitivity analysis, we additionally adjusted for seasonality (defined using sine and cosine functions)[26] to further address residual confounding, and our conclusions remained the same (data not shown). In the $PM_{2.5}$-HRV analysis, we additionally adjusted for HR to investigate if the change in HRV was partially due to HR fluctuation, and we obtained similar results (data not shown). We also ruled out confounding due to dietary intake of folic acid, vitamin B_6 and B_{12}, as no significant changes was observed during the study period (Supplementary Table 1.1).

Supplementary Table 1.1 Dietary intake of folic acid, vitamin B_6 and B_{12}, at screening and the end of study

	Mean (SD)	Median (IQR)	Range	Mean (SD)	Median (IQR)	Range	*P* for T test
		Screening			End of Study		
Folic acid(mcg/d)	172.8 (105.7)	161.5 (143.0)	17.0 to 376.0	171.8 (121.7)	169.0 (130.0)	3.0 to 429.0	0.98
B_6 (mg/d)	2.1 (0.8)	2.2 (0.6)	0.3 to 3.6	2.0 (0.7)	2.0 (1.1)	0.9 to 3.2	0.86
B_{12} (mcg/d)	6.5 (4.1)	5.6 (4.1)	0.9 to 13.4	5.6 (2.2)	6.8 (2.5)	1.3 to 7.6	0.57

SD indicates standard deviation; IQR indicates inter-quartile range.

DISCUSSION AND CONCLUSION

This single-blind crossover intervention trial with controlled exposure experiments found that two-hour exposure to concentrated ambient $PM_{2.5}$ ($250\mu/m^3$) has substantial physiologic impacts on HR, HRV, and WBC among healthy adults. Furthermore, we demonstrated that these effects are nearly entirely abolished with four-weeks of B vitamin supplementation.

Ambient $PM_{2.5}$ pollution continues to be a major cardiovascular public health challenge facing the world today, with levels of pollution that are far higher than U.S. EPA National Ambient Air Quality Standards (NAAQS) in many urban megacities world-wide.[27] Regulation remains the backbone of public health protection against cardiovascular health effects of particle pollution. Improvement in cardiovascular health with reduction in morbidity and mortality has been documented as pollution levels have decreased in the U.S.[28] Nevertheless, even in U.S. cities in compliance with NAAQS, cardiovascular effects of particle pollution have been documented with no evidence for a threshold for effect in sensitive individuals.[3,4,6,27] Thus the physician and public health community have sought adjunct personal measures that might complement regulation in reducing the cardiovascular risk of pollution in sensitive people.[29]

Our choice to assess the potential benefits of B vitamin supplementation was motivated by the anti-inflammatory, antioxidant, and epigenetic effects of B vitamins.[30,31] In addition, some observational and laboratory studies suggest that B vitamins might be particularly protective against air pollution-induced cardiovascular outcomes, and intermediate pathway biomarkers.[6,17,30,31] For example, folic acid and vitamin B_6 lowers circulating levels of pro-inflammatory molecules,[30,31] and attenuates lipopolysaccharide-induced nuclear factor-kβ

pathway activation.[16] In addition, adequate B vitamin intake ensures proper epigenetic status in WBCs – which warrants proper immuno-regulation and prevents excessive oxidative damage to the cardiovascular system.[3,4] Although the results of randomized controlled trials on supplementation with folic acid, vitamin B_6 and B_{12} do not support benefits of B vitamin supplement for either primary or secondary CVD prevention,[12,14,15,32] the abovementioned interactive biological properties of B vitamins render it a promising preventive strategy to minimize the cardiovascular damage due to ambient $PM_{2.5}$ pollution. However, no prior clinical investigation has tested whether B vitamins supplementation can be used to guard the cardiovascular system from the adverse health effects of $PM_{2.5}$.

Primary autonomic and WBC influences of $PM_{2.5}$

Our findings of a primary autonomic effect of $PM_{2.5}$ are consistent with previous human controlled exposures studies,[33-35] showing that short-term $PM_{2.5}$ exposure perturbed cardiorespiratory autonomic function as reflected in increased HR and reduced HRV.[6] Immediately following two-hour exposure to ambient concentrated $PM_{2.5}$, we observed a substantial increase in resting HR (3.8 beat/min) and a 57.5% reduction in LF power, compared to medical air. Our results indicate a consistent reduction in HRV across five measures – which reflects the adverse pathophysiological modulations in cardiac autonomic control by $PM_{2.5}$ exposure.

$PM_{2.5}$ is a potent trigger for leukocyte-mediated inflammation, which is proposed to be the key mechanism underlying the pathological modulation of the cardiovascular system by $PM_{2.5}$ exposure.[4] Our data supports this hypothesis by showing that two-hour $PM_{2.5}$ exposure

immediately triggers a 9.9% increase in total WBC count and a 10.0% increase in lymphocyte count (Table 1.6). In healthy adults, PM pollution has been shown to increase the number of neutrophil and lymphocyte in alveolar lavage and peripheral blood.[36] While the underlying biological mechanism remains unclear, *in vivo* studies suggested that PM stimulates bone marrow via alveolar macrophages-mediated cytokine signaling, leading to accelerated release of immature leukocytes in to the circulation.[37,38]

Twenty-four hours after the exposure, the effect of $PM_{2.5}$ on HR and HRV weakened (Table 1.5). However, $PM_{2.5}$ exposure remained significantly associated with >10% higher numbers of total WBC and lymphocyte (Table 1.6). Taken together, although the acute physiological responses due to $PM_{2.5}$ exposure peak might be reversible, the pro-inflammatory effects of $PM_{2.5}$ appears to be sustained beyond 24 hours and represent a biomarker of a process that could have clinical relevance to sensitive individuals in a community setting.[4]

Modifying of $PM_{2.5}$ autonomic and WBC effects by B vitamins

For the first time, our trial provided evidences demonstrating the unique preventive benefits of B vitamin administration in the context of air pollution: B vitamin supplements can diminish the acute effects of $PM_{2.5}$ on a battery of cardiac autonomic dysfunction measures and inflammatory markers. These findings are in line with our results from the Normative Aging Study[6] – a population with average B vitamins intakes well above the standard dietary references – short-term $PM_{2.5}$ exposure was associated with lower HRV (7.1% reduction in SDNN per 10 $\mu g/m^3$ increase in $PM_{2.5}$), and this effect was limited to subjects with lower (<median) intakes of vitamin B_6, vitamin B_{12}, or methionine.

This study has several strengths, including its crossover design with controlled exposure experiments – which simulate conditions similar to urban air pollution peaks, while allowing for control of exposure and treatment at the individual level. The Harvard ambient particle concentrators do not affect the concentration of gaseous pollutants, therefore, minimizing the confounding due to gaseous co-pollutants such as ozone and sulfur dioxide. All exposure experiments were conducted at the same time of the day to eliminate impact due to diurnal variation. We adjusted for time-varying factors including season, chamber temperature and humidity to minimize their influence on the observed associations, while time invariant factors are controlled by the crossover design.

We acknowledge several limitations in the present study. Our study is underpowered to detect small effects with only 10 subjects (30 controlled exposure experiments). The present study could not randomize the treatment sequence due to the long half-life of B vitamins, therefore might be subject to confounding by period or ordering effects. For example, as the study progressed, the volunteers might be more tolerant with $PM_{2.5}$ exposure because of learning effect. We intentionally provided medical air as the first exposure experiment; therefore the learning effect is expected to bias the effect of $PM_{2.5}$ towards the null. The short study duration with only four-week time intervals between exposure experiments also reduced the impact of temporal trend. In addition, we contrasted the post- *vs* pre-exposure status to ascertain all outcome measurements, which is expected to be less prone to confounding due to temporal trend than the absolute values. While residual confounding is possible, considering the magnitude of our effect

estimates and the consistency across different HRV index, it is unlikely that the observed association reflected bias resulting from confounding.

Apart from avoiding exercising outdoors at peak pollution times, sensitive individuals have limited personal options to reduce exposure and hence cardiovascular risk from ambient $PM_{2.5}$ pollution. While regulation is the backbone of prevention, residual risk remains for those who are sensitive, and high exposures are, unfortunately, the rule still in many megacities throughout the world. The present study for the first time provide experimental evidence showing that ambient $PM_{2.5}$ exposure peak has unfavorable effect on cardiac autonomic function and the immune system, which, can be counteracted by B vitamin supplementation. Our project inaugurated a new line of research for the development of preventive pharmacological interventions to curb the health effect of air pollution. Future studies are warranted to identify the precise pathophysiological processes of PM-induced cardiovascular responses and inflammation, as well as the mechanistic pathway underlying the protective effect of B vitamins.

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Chapter 2

B Vitamins Curbs the Epigenetic Effect of Concentrated Ambient Fine Particles: An Epigenome-wide Association Study in a Human Intervention Trial

Background: Fine particulate matter ($PM_{2.5}$) pollution alters DNA methylation status, a reversible epigenetic process underlying PM-induced inflammation and oxidative stress. We aimed to determine the genome-wide epigenetic effects of PM, and whether B vitamins supplement can avert these effects.

Methods/Results: We conducted a crossover intervention trial with ten healthy adults. Each participant received placebos and then four-week B vitamins supplements (2.5 mg/d folic acid, 50 mg/d vitamin B_6, and 1 mg/d vitamin B_{12}). Three two-hour controlled exposures to medical air or $PM_{2.5}$ ($250\mu g/m^3$) were administered in a pre-determined order. We measured the DNA methylation profile, pre and post each exposure, using the Infinium HumanMethylation450 BeadChip in peripheral CD4+ T helper (T_h) cells. Among ten top loci that were associated with PM exposure, two were involved in mitochondria oxidative metabolism. The effects of PM on these loci were attenuated with B vitamins supplement (P=0.003; P=0.01; P=0.0007; P=0.13; P=0.01; P=0.001; P=0.004; P=0.0004; P=0.0002; P=0.07). Motivated by these findings, we further assessed mitochondrial DNA content in CD4+ T_h cells using quantitative polymerase chain reaction. At 24-hour post exposure, $PM_{2.5}$ was associated with 8.11% (95% CI, 3.31%, 12.91; P=0.002) lower mitochondrial DNA content compared to medical air. Effect of $PM_{2.5}$ were abrogated with B vitamins supplement – which attenuated the association of $PM_{2.5}$ with mitochondrial DNA content by 105% ($P_{interaction}$=0.02).

Conclusions: In healthy adults, two-hour $PM_{2.5}$ exposure substantially modulated the DNA methylation landscape and resulted in mitochondrial DNA depletion in circulating CD4+ T_h cells. These effects can be mitigated with B vitamins supplement.

INTRODUCTION

Ambient $PM_{2.5}$ (particles with an aerodynamic diameter <2.5 micrometers) pollution, a major public health challenge facing the world today,[1, 2] accounts for 3.7 million premature deaths per year.[1] $PM_{2.5}$ penetrates into the lower airways and chronically deposits in the respiratory bronchioles and the alveoli, stimulating local and systemic inflammation and oxidative stress.[3, 4] Each 10 $\mu g/m^3$ elevation in annual $PM_{2.5}$ exposure has been associated with an 11% increase in mortality.[5] Over the past few decades, substantial lowering of ambient $PM_{2.5}$ levels has been achieved by massive emission control efforts.[6] However, exposure peaks with adverse health consequences are still frequently recorded in the United States,[7, 8] even in areas with low usual levels.[9] While the mechanism underlying $PM_{2.5}$'s health effects remains the major gap in current knowledge, the lack of preventative options adds another layer of complexity to tackle this major public health challenge.

Recent studies on environmental epigenetics provide tremendous opportunities to understand the mechanistic underpinning, and to develop novel interventions to be used at the personal level. DNA methylation, a reversible epigenetic mechanism, regulates gene expression via addition of methyl groups to the cytosine.[10] Exposure to $PM_{2.5}$ can rapidly alter the dynamic DNA landscape in peripheral leukocytes, which is postulated to underlie PM-induced systemic inflammation and oxidative stress.[11-14] While most available evidence derives from human studies are based on a heterogeneous mixture of leukocytes,[15-17] *in vivo* studies reported loss of methylation in inflammatory genes and subsequent inflammatory responses specifically in circulating T helper (T_h) cells, after environmental challenge.[18, 19]

Notably, DNA methylation is dependent on a biochemical cycle that supplies methyl groups (CH_3) while relying on methyl nutrients (i.e., B vitamins including folic acid, vitamin B_6 and B_{12}; and amino acids including methionine, betaine, and choline).[10, 20, 21] In animal studies, a diet deficient in methyl nutrients leads to aberrant DNA methylation patterns,[22, 23] and administration of methyl nutrients enables restoration of epigenetic status.[14, 20, 24-27] Likewise, human studies showed the plasticity of DNA methylation in response to dietary methyl nutrients intervention.[28] Potential for epigenetic modulation was also demonstrated in the presence of environmental stressors – Dolinoy and coauthors successfully used methyl nutrients to avert the DNA hypomethylation induced by exposure to Bisphenol A.[29] These findings have opened new avenues for the application of epigenetic intervention to reduce the health effects of air pollution. However, to date, human intervention study with epigenetic components in the context of air pollution is lacking.

The present study is the first crossover intervention trial with controlled human exposure to fine concentrated ambient particles (fine CAP, or $PM_{2.5}$). We hypothesized that acute $PM_{2.5}$ exposure can rapidly modify the DNA methylation profile in CD4+ T_h cells – the most prolific cytokine producer that mediates PM-induced inflammatory responses[24,25] – and these effects can be curbed by intervention with B vitamins (i.e., folic acid, vitamin B_6 and B_{12}), an important source of methyl groups (Figure 2.1).

METHODS

Study population

We recruited ten healthy, 18–60 year old, non-smoking volunteers from the University of Toronto campus and surrounding area. All volunteers were not taking any medicines or vitamin supplements for at least more than four weeks before the enrollment. The study was approved by all participating institutes and registered. We obtained written informed consent from every volunteer before enrolling.

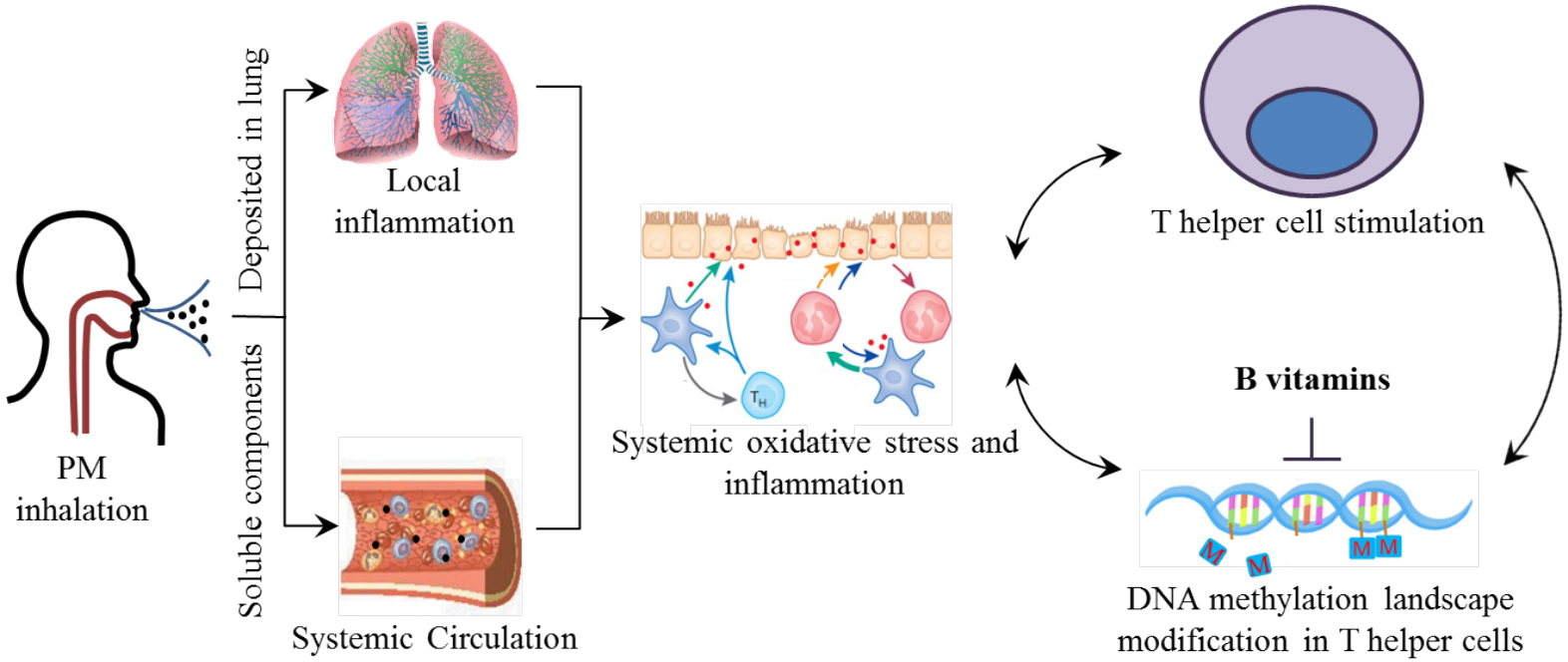
Study design

We conducted a single-blind, crossover, intervention trial with controlled exposure to concentrated ambient $PM_{2.5}$ (July 2013 to February 2014). The design started with a two-week run-in period with placebo and then the baseline blank study (two-hour exposure to particle-free medical air, exposure one). After exposure one, each volunteer took placebo for four weeks and then exposed to $PM_{2.5}$ (two hours, target concentration: 250 $\mu g/m^3$, exposure two). All volunteers started the four-week B vitamins supplement right after exposure two, and then were exposed again to $PM_{2.5}$ exposure (two hours, target concentration: 250 $\mu g/m^3$, exposure three).

Exposure facility

We used the Harvard ambient particle concentrator, a 3-stage high-flow (5,000 L/min) virtual impactor, to generate concentrated ambient $PM_{2.5}$.[30] Ambient particles were drawn in from an inlet 1.5 meters high, beside a busy (>1,000 vehicles/hour) street in downtown Toronto. The $PM_{2.5}$ air stream is delivered directly to the volunteer who is seated inside a 4.9 m^3 (1.1 x 1.9 x 2.0 m) lexan enclosure, at rest and breathing freely via an "oxygen type" facemask on his/her

Figure 2.1 Proposed conceptual model linking fine particulate matter ($PM_{2.5}$) exposure, systemic oxidative stress and inflammation, and altered DNA methylation landscape in T helper cells



nose and mouth. The Medical air exposures were generated as previously described.[31] During each $PM_{2.5}$ exposure experiment, particles were collected on polycarbonate membrane filters. The gravimetric determination of $PM_{2.5}$ exposure mass concentration ($\mu g/m^3$) was monitored.

Folic acid, vitamin B_6, and vitamin B_{12} supplement

We administered one placebo or B vitamins supplement (2.5 mg folic acid, 50 mg vitamin B_6, and 1 mg vitamin B_{12}) daily to each volunteer. Preparation and packaging of the placebo and B vitamins supplement was done by an external lab (Jamieson Laboratory, Toronto, Canada) to ensure blinding. The coding used on the label was blinded to the volunteers and investigators. We monitored volunteers' plasma folic acid, vitamin B_6 and B_{12} levels prior to each exposure experiment. In addition, we assessed typical daily dietary intake of nutrients with a self-administered validated semi-quantitative Food Frequency Questionnaire (FFQ) at the first and the last visit, to rule out potential impact from diet.

T-helper cells isolation and DNA extraction

We collected blood samples via venous phlebotomy, and within four hours, isolated CD4+ T_h cells using negative selection (i.e. removal of other cell types). We targeted and removed unwanted cells using the RosetteSep® Human CD4+ T Cell Enrichment Cocktail (Stem Cell Technologies, #15062), without affecting CD4+ T_h cells. DNA was extracted from isolated CD4+ T_h cells using a Promega Maxwell 16 tissue DNA purification kit with a Promega Maxwell 16 instrument (Promega, Madison, WI). We monitored the concentration and quality of extracted DNA using NanoDrop ND-1000 spectrophotomer (Nanodrop Technologies, Oxfordshire, UK). Unsatisfactory DNA samples were discarded and re-extracted.

Epigenome-wide DNA methylation scan

We diluted purified DNA samples to a concentration of 30 ng/ul, with a total volume of 40 ul (total amount: 1200 ng) and sent the de-identified samples to an external lab (Genomics Core Facility, Northwestern University) for bisulfite conversion and hybridization on the Infinium Human Methylation 450K BeadChip (Illumina, San Diego, CA, USA).[32] The 450K BeadChip allows the assessment of approximately half a million CpG sites across 99% of RefSeq genes within the genome.[16, 33, 34] Because of the within-subject cross-over design, we plated all samples from one subject in one chip, with pre- and post-exposure samples randomly loaded onto each column on the same row. All samples were processed by one technician and analyzed in one batch to minimize batch effect. We obtained the β-value for each CpG, derived from the fluorescent intensity ratio (β = intensity of the methylated allele/(intensity of the unmethylated allele + intensity of the methylated allele + 100)).

Quality control for DNA methylation data

Systematic QA/QC was performed, including assessment of assay repeatability and chip/plate effects using 10 across-chip technical replicates. We preprocessed the IDAT files using minfi package.[35] We screened for signal outliers using multidimensional scaling analysis and no outlier were identified. We dropped the probes that are: 1.) where 20% of samples had detection P-values >0.01 were dropped (n=441); 2.) assay SNPs rather than CpGs (n=65); 3). located on X or Y chromosome (n=11341). After excluding these probes, as well as control probes and probes on sex chromosomes, a total of 473,730 CpGs were analyzed. All samples had > 95% sites with detection $p < 0.01$. We checked gender concordance and did not observe any sample mismatch.

We minimized color channel bias and difference in Infinium chemistry by using dasen method in wateRmelon package to normalize[36] – which equalized type I and type II backgrounds first, and then quantile-normalized type I and type II intensities separately.

Mitochondrial DNA content in CD4+ T_h cells

Motivated by the top loci associated with $PM_{2.5}$ exposure, we measured mitochondrial DNA content in CD4+ T_h cells through the mtDNA/nDNA ratio, a widely used biomarker representing the mitochondrial DNA copy number versus the nuclear DNA copy number.[37, 38] Mitochondria DNA copy number was analyzed pre, post, and 24-hour post each exposure experiment using multiplex quantitative real-time polymerase chain reaction, as previously reported.[39] The mtDNA/nDNA is used in the statistical analysis – a ratio value of 1 indicates that the mtDNA/nDNA of the test sample is equal to the mtDNA/nDNA in the reference DNA pool used in the assay.

Statistical methods

Covariates Selection and Model Assumption

The crossover design minimized the influence from time-invariant factors (such as age, gender, race, etc.). In all models, we adjusted for covariates with potential influences on DNA methylation, selected based on prior knowledge and the existing literature, i.e., season (fall/winter/spring/summer), chamber temperature, and relative humidity.[4, 31]

Linear mixed-effects models

Rank-based normal transformation was performed on all DNA methylation measures to improve normality and stabilize the variance.[19] In the CpG-by-CpG analyses, we used linear mixed-effects models **(Model 1)** to account for within-subject correlation in methylation measures. Random intercepts were assigned to each subject.

$$Y_{ij} = \beta_0 + \beta_1 PM_{ij} + \beta_2 PM_{ij}*Treatment_{ij} + \beta_3 X_{1ij} + \ldots + \beta_p X_{pij} + b_i + \varepsilon_{ij} \quad \text{(Model 1)}$$

In the above model, Y_{ij} was the β value of the measured CpG probe for participant *i* at exposure occasion *j*, β_0 was the overall intercept, and b_i was the separate random intercept for subject *i* with, $b_i \sim N(0, \Theta)$, $\varepsilon_{ij} \sim N(0, \sigma^2)$. X_{1ij}–X_{pij} were the covariates, for participant *i* at measurement *j*. The main effect of B vitamin supplementation was not included in the model, given volunteers did not receive any medical air exposure while on B vitamin supplementation. β_1 represents the effect of $PM_{2.5}$ exposure without B vitamin supplementation and $\beta_1 + \beta_2$ represents the effect of $PM_{2.5}$ exposure with B vitamin supplementation. β_2 thus represents the intervention effect of B vitamin supplementation (i.e, the attenuation of $PM_{2.5}$ effect due to B vitamin supplementation).

In addition, we used another linear mixed-effects model **(Model 2)** as secondary analysis to estimate the main effect of B vitamin supplementation assuming equivalent outcome status prior to exposure two and three.

$$Y_{ij} = \beta_0 + \beta_1 Post_{ij} + \beta_2 Post_{ij}*PM_{ij} + \beta_3 Treatment_{ij} + \beta_4 Treatment_{ij}*Post_{ij} + \beta_p X_{1ij} \ldots + \beta_p X_{pij} + b_i + \varepsilon_{ij} \quad \text{(Model 2)}$$

For participant *i* at exposure occasion *j*, $Post_{ij}$ (binary) indicates whether the methylation profile is measured pre- or post- exposure experiment; PM_{ij} (binary) indicates exposure to $PM_{2.5}$ or medical air; $Treatment_{ij}$ (binary) indicates placebo or B vitamins supplement; X_{1ij}–X_{pij} were the covariates. β_1 represents the effect of exposure to medical air; β_2 represents the effect of exposure

to $PM_{2.5}$, compared to medical air; β_3 represents the effect of B vitamins supplement; β_4 represents the change in $PM_{2.5}$ effect (comparing to medical air) when B vitamins supplement is added.

Analyses were performed using SAS 9.4 (SAS Institute, Cary NC) and R statistical computing software (R Foundation for Statistical Computing, Vienna, Austria). Top associated loci were defined based on the effect size and P value of β_2. The linear mixed-effects model was implemented using the lme4[40] and lmerTest[41] packages.

We estimated the proportions of major leukocyte types (CD4 T cells, CD8 T cells, B Cells, granulocytes, monocytes, and natural killer cells) to assess the purity of isolated CD4+ T_h cells using the Houseman method, a statistical deconvolution technique based on the 450K data.[15]

RESULTS

Study population, plasma B vitamins concentrations, and exposure levels

Ten volunteers completed 30 controlled exposure experiments in total (10 medical air exposures and 20 $PM_{2.5}$ exposures). Forty percent of the volunteers were white, 30% were Asian, and 30% were other races. Sixty percent of the volunteers were female and 30% had BMI ≥25 (Table 2.1). Table 1 presents the distributions of baseline mitochondrial DNA content across subgroups.

We targeted the concentrations of $PM_{2.5}$ at 287.5 $\mu g/m^3$; however, the actual $PM_{2.5}$ mass concentration varied among exposure experiments. In all controlled exposure experiments to $PM_{2.5}$, the $PM_{2.5}$ concentration varied from 100.6 $\mu g/m^3$ to 287.5 $\mu g/m^3$ with a median of 234.0 $\mu g/m^3$. There was no significant difference in $PM_{2.5}$ concentration between exposure one and two (P=0.38).

Prior to medical air exposure, the mean plasma concentration of folic acid, vitamin B_6 and B_{12} was 37 nmoles/L, 42 nmoles/L, and 305 pmoles/L, respectively. After taking placebos for four weeks, the mean plasma concentrations remained similar: 39 nmoles/L for folic acid (P=0.82), 87 nmoles/L for vitamins B_6 (P=0.75), and 305 pmoles/L for vitamins B_{12} (P=0.42). However, vitamins supplement significantly increased the mean plasma concentrations of folic acid (124 nmoles/L, P=0.02), vitamin B_6 (393 nmoles/L, P=0.004), and vitamin B_{12} (507 pmoles/L, P=0.01).

CD4+ T_h cells purity

Table 2.1 Baseline characteristics of the study participants (N=10)

Characteristics	N (%)	mtDNA copy number		
		Mean (SD)	Minimum	Maximum
Age (years)				
19-29	7 (70)	1.6 (0.1)	1.4	1.8
30-49	3 (30)	2.0 (0.4)	1.6	2.4
Race				
White	4 (40)	1.9 (0.3)	1.6	2.4
Asian	3 (30)	1.5 (0.0)	1.5	1.6
Other	3 (30)	1.6 (0.2)	1.4	1.7
Gender				
Female	6 (60)	1.6 (0.1)	1.5	1.8
Male	4 (40)	1.8 (0.4)	1.4	2.4
BMI (kg/m^2)				
<25	7 (70)	1.6 (0.1)	1.4	1.8
≥25	3 (30)	2.0 (0.4)	1.5	2.4

mtDNA indicates mitochondrial DNA; SD indicates standard deviation; BMI indicates body mass index.

Based on the Houseman cell proportion estimates, all CD4+ T_h cell samples' purity were over 80% (Table 2.2). The median purity of CD4+ T_h cell samples collected at exposure one, exposure two, and exposure three was 96.92%, 94.84%, and 96.09%, respectively. There was only minor contamination from CD8 T cells, B cells, granulocytes, and natural killer cells.

Effect of $PM_{2.5}$ and B vitamins supplement on DNA methylation

We observed substantial modification of the DNA methylation landscape associated with $PM_{2.5}$ exposure and B vitamins supplement (Figure 2.2). Acknowledging that the present study is limited in power with only ten volunteers, we presented the top ten loci selected based on both effect size and P value (Table 2.3 and Figure 2.2), although no locus reached the Bonferroni threshold of significance.[42] In the absence of B vitamins supplement, $PM_{2.5}$ exposure for two hours was associated with both increased and decreased DNA methylation level compared to medical air exposure, immediately after exposure (Table 2.3). Quantile-Quantile plots for expected vs. observed distribution of P values showed minimal genomic inflation (Figure 2.3). Among the ten CpG sites, three were located on chromosome 2, two were located on chromosome 6, two were located on chromosome 19, and one each located on chromosomes 12, 18, and 21. Five of the top loci were positively associated with exposure to $PM_{2.5}$, while the other five top loci were negatively associated with exposure to $PM_{2.5}$ (Table 2.3).

Four-week B vitamins supplement attenuated the association of $PM_{2.5}$ with all top loci ($P_{interaction}$=0.003; $P_{interaction}$=0.01; $P_{interaction}$=0.0007; $P_{interaction}$=0.13; $P_{interaction}$=0.01; $P_{interaction}$=0.001; $P_{interaction}$=0.004; $P_{interaction}$=0.0004; $P_{interaction}$=0.0002; $P_{interaction}$=0.07). For example, supplementing B vitamins resulted in a reduction in effect size by 58% for cg06194186,

Table 2.2 The Houseman estimates of different leukocyte types in isolated CD4+ T helper cells

Cell Type	Median	IQR	Minimum	Maximum
		Exposure 1		
CD4 T helper Cells (%)	96.92%	5.12%	81.85%	100.00%
CD8 T Cells (%)	2.67%	3.74%	0.00%	5.92%
B Cells (%)	0.30%	1.13%	0.00%	8.20%
Granulocytes (%)	0.00%	0.20%	0.00%	4.65%
Monocytes (%)	0.00%	0.00%	0.00%	0.00%
Nature Killer Cells (%)	0.00%	0.00%	0.00%	4.68%
		Exposure 2		
CD4 T helper Cells (%)	94.84%	7.14%	81.03%	99.65%
CD8 T Cells (%)	3.14%	2.46%	0.00%	6.01%
B Cells (%)	0.75%	2.88%	0.00%	5.20%
Granulocytes (%)	0.00%	0.87%	0.00%	4.90%
Monocytes (%)	0.00%	0.00%	0.00%	0.00%
Nature Killer Cells (%)	0.00%	0.00%	0.00%	7.65%
		Exposure 3		
CD4 T helper Cells (%)	96.09%	4.73%	85.10%	100.00%
CD8 T Cells (%)	2.37%	4.04%	0.00%	5.77%
B Cells (%)	0.42%	1.04%	0.00%	3.94%
Granulocytes (%)	0.00%	0.21%	0.00%	6.14%
Monocytes (%)	0.00%	0.00%	0.00%	0.00%
Nature Killer Cells (%)	0.00%	0.00%	0.00%	3.11%

IQR indicates inter-quartile range.

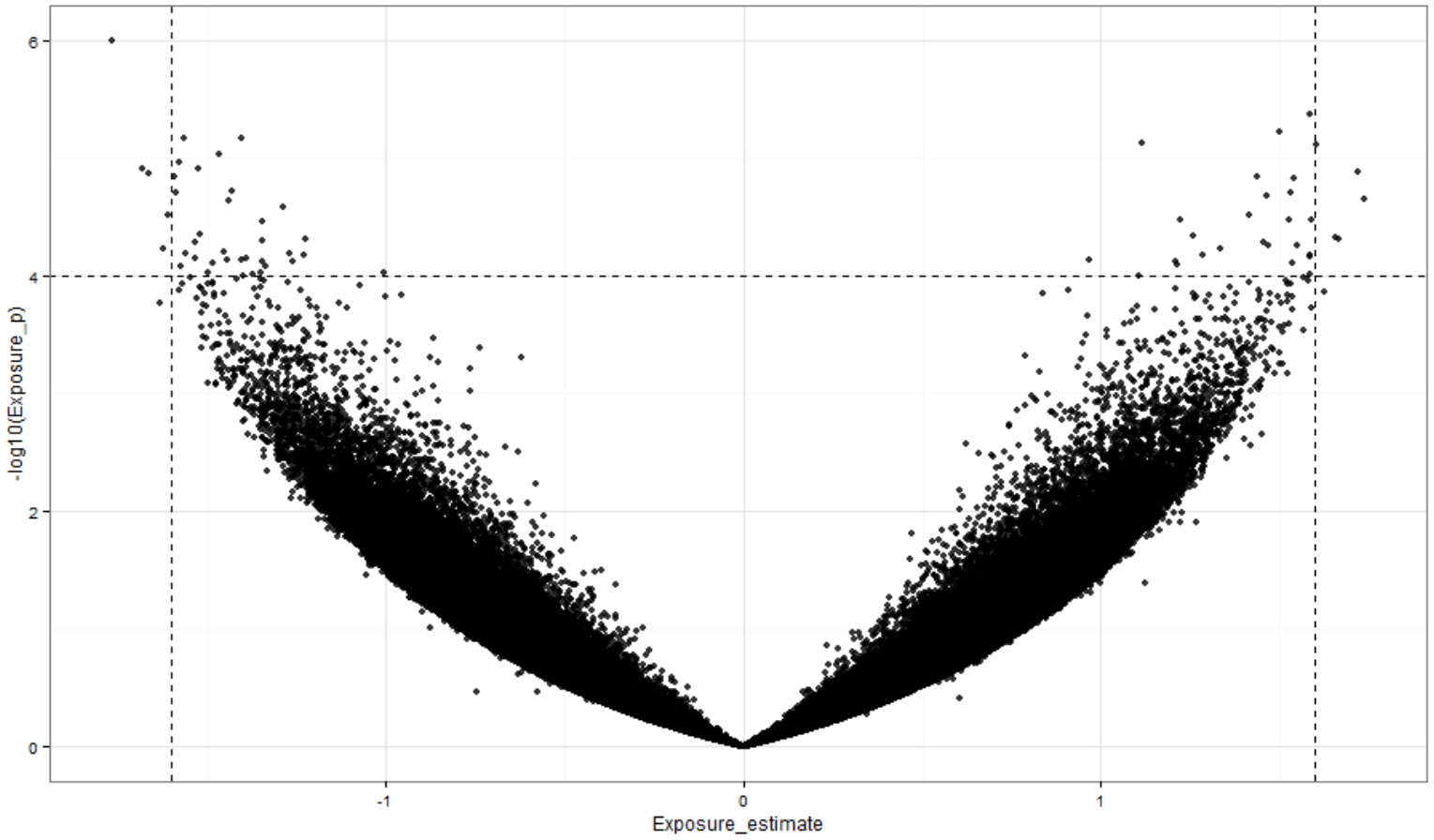
Table 2.3 Top ten loci associated with two-hour PM exposure and the effect modification by B vitamins supplement, selected by effect size

Illumina CpG designation	Genomic position	Relation to CpG islands	Gene symbol	Change in DNAm β	*P*	Effect modification by B vitamins	*P*
Positive association with $PM_{2.5}$ exposure							
cg06194186	Chr 2: 207803693		CPO	1.74	2.3E-05	-1.00	0.003
cg07689821	Chr 6: 33290736	Island	DAXX	1.72	1.3E-05	-0.84	0.01
cg00068102	Chr 2: 121619261		GLI2	1.67	4.9E-05	-1.22	0.0007
cg00647528	Chr 18: 32957127	Island	ZNF396	1.66	4.7E-05	-0.52	0.1
cg15426626	Chr 12: 132984261†	Island		1.60	7.6E-06	-0.73	0.01
Negative association with $PM_{2.5}$ exposure							
cg10719920	Chr 21: 46724843†	S_Shelf		-1.77	1.0E-06	0.49	0.07
cg21986027	Chr 6: 169238138†			-1.68	1.2E-05	1.27	0.0002
cg17157498	Chr 19: 1383493	Island	NDUFS7	-1.67	1.3E-05	1.24	0.0004
cg08075528	Chr 19: 1961017	Island	CSNK1G2	-1.63	5.8E-05	1.03	0.004
cg26995744	Chr 2: 200322034	Island	SATB2	-1.61	3.0E-05	1.14	0.001

SD indicates standard deviation; IQR indicates inter-quartile range.

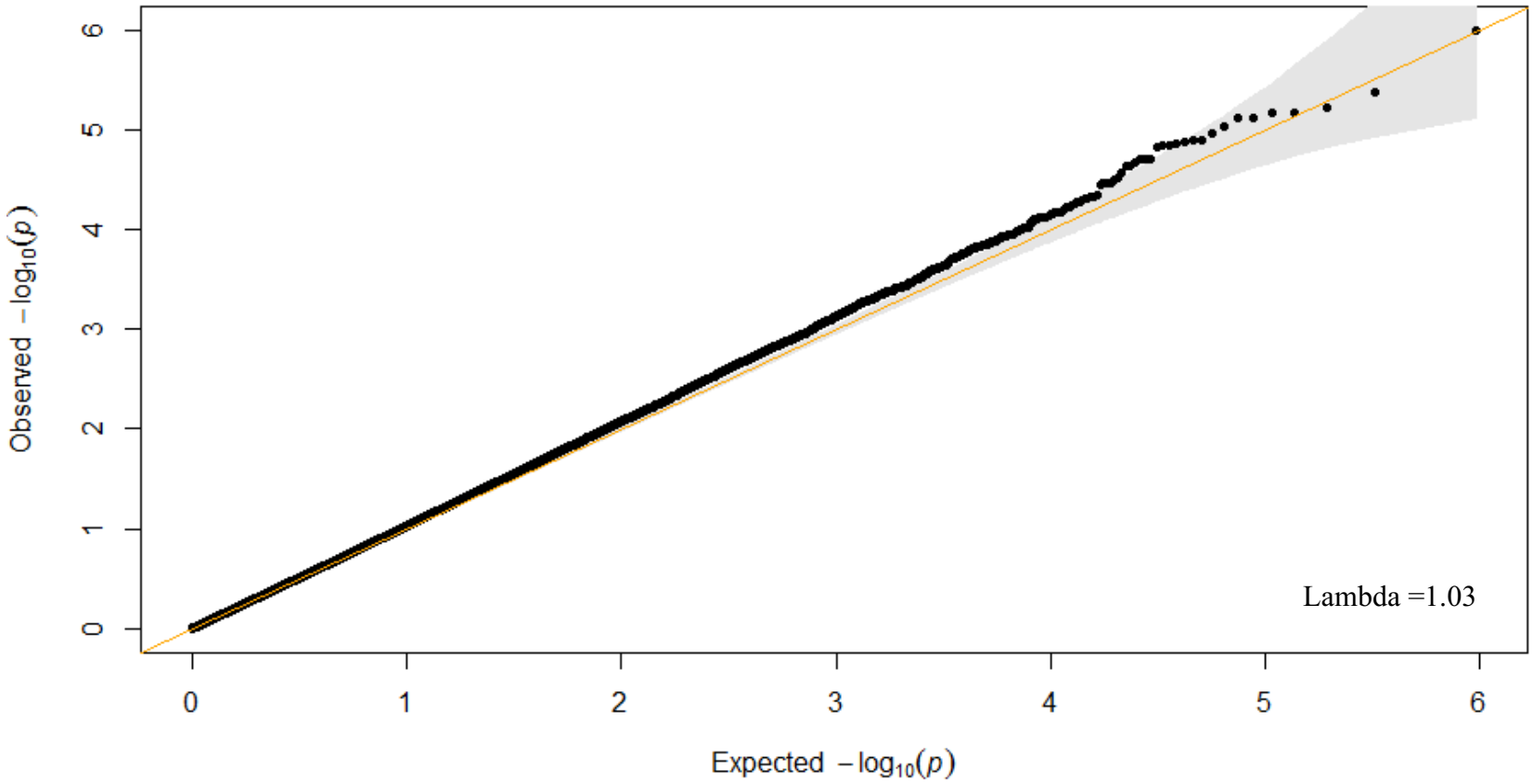
† Illumina array annotation indicates that these loci are not located within an annotated CpG region and are not associated with any gene.

Figure 2.2 Volcano plot depicting the distribution of estimated effect of fine particle exposure on the epigenome



Infinium 450K data were filtered for poor-performing samples and probs and normalized. Analyses were adjusted for season, chamber humidity and temperature. The vertical lines indicate suggestive threshold based on effect size, while the horizontal line reflects the suggestive threshold based on effect size and statistical significance (P values).

Figure 2.3 Quantile-Quantile plot for associations of fine particle exposure with epigenome-wide DNA methylation in circulating CD4+ T helper cells



49% for cg07689821, 73% for cg00068102, 31% for cg00647528, 45% for cg15426626, 28% for cg10719920, 75% for cg21986027, 74% for cg17157498, 63% for cg08075528, and 71% for cg26995744, respectively.

The genomic position, relation to CpG islands, and gene symbol are listed in detail in Table 2.3. The top two loci that are associated with known genes (selected based on effect size of $PM_{2.5}$) were cg06194186 and cg17157498. Locus cg06194186 is located in the promoter region (TSS1500) of *CPO* (*Carboxypeptidase O*) gene, and locus cg17157498 is located in the promoter region (TSS1500) of *NDUFS7* (*NADH Dehydrogenase (Ubiquinone) Fe-S Protein 7*) gene.

Effect of $PM_{2.5}$ and B vitamins supplement on mitochondrial DNA content

Because *CPO* and *NDUFS7* are both involved in mitochondrial oxidative metabolism,[43, 44] we further tested the associations of $PM_{2.5}$ with mitochondrial DNA content (mtDNA/nDNA ratio), as well as the potential role of B vitamins supplement on their relationship. Compared to medical air, two-hour exposure to $PM_{2.5}$ was estimated to be nonsignificantly associated with an 1.14% (95%CI: -8.71%, 10.99%; P=0.82) change in mitochondrial DNA content. However, 24 hours after exposure, $PM_{2.5}$ exposure was significantly associated with 8.11% (95%CI: 3.31%, 12.91%; P=0.002) lower mitochondrial DNA content, compared to medical air (Table 2.4). We did not observe significant effect of medical air on the mitochondrial DNA content (Table 2.4). B vitamins supplement did not seem to have direct effect on the mitochondrial DNA content; however, it significantly reduced the estimated effect of $PM_{2.5}$ on the mitochondrial DNA content by 105% ($P_{interaction}$=0.02), at 24-hour post exposure (Figure 2.4 and Table 2.4). With B vitamins supplement, two-hour $PM_{2.5}$ exposure was no longer significantly associated with

mitochondrial DNA content (95%CI: -10.80%, 7.63; P=0.73) (Table 2.4) change in the. Likewise, B vitamins supplement nonsignificantly reduced the estimated effect of $PM_{2.5}$ on the mitochondrial DNA content by 47% ($P_{interaction}$=0.93), immediately post exposure (Figure 2.4 and Table 2.4).

Sensitivity analysis

We conducted permutation test to rule out that the observed top two loci were false-positive finding. We randomly paired the exposure and treatment status with methylation data within each subject and ran liner mixed models with random intercepts. We calculated the permutation P value testing being a false-positive finding (H_0) based on the observed t statistic (t.actual) and the distribution of the t statistics obtained from 1000 permutation simulations (t.nulls). When t.actual is greater than 0, the $P_{permutation}$ is defined as (number of t.nulls > t.actual + number of t.nulls < -t.actual)/1000. When t.actual is less than 0, the $P_{permutation}$ is (number of t.nulls < t.actual + number of t.nulls > -t.actual)/1000. The $P_{permutation}$ was <0.0001 for both top loci.

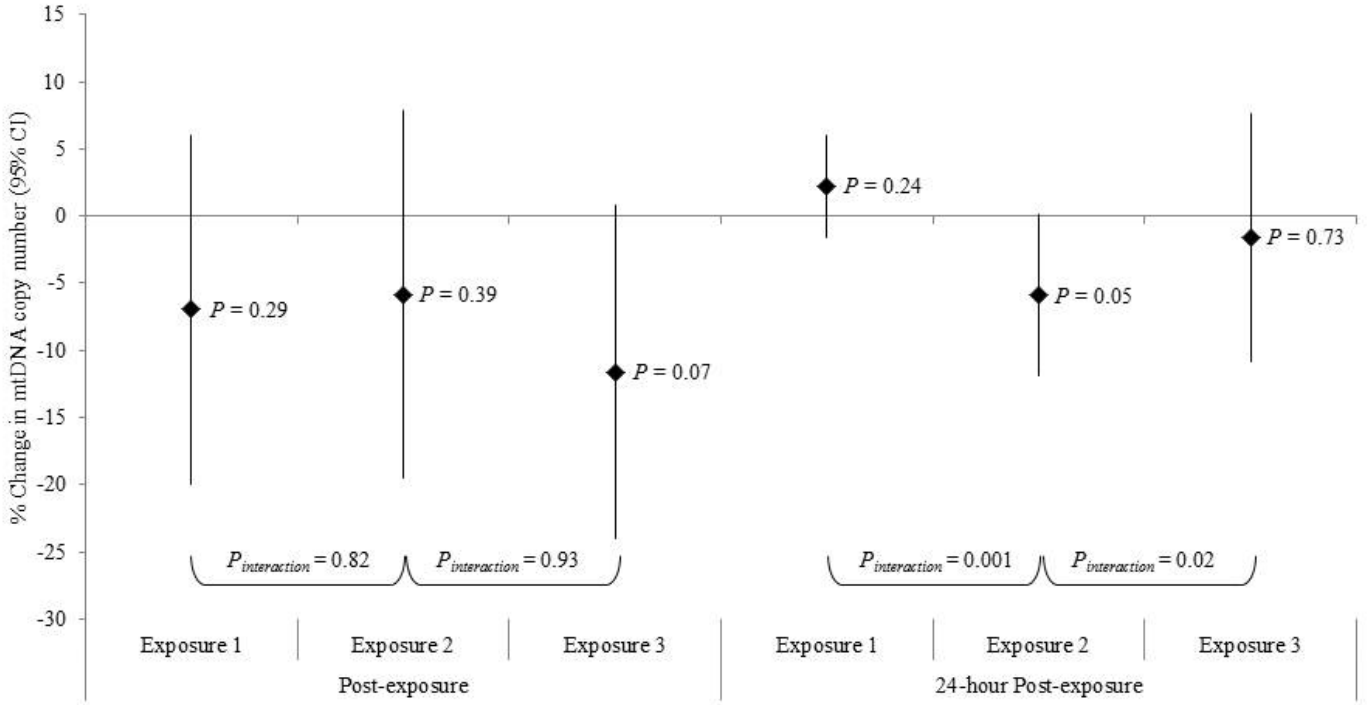
Due to long biological half-life of body stores of B vitamins,[45] the present study could not randomize on the order for placebo *vs* B vitamins supplement. This created potential confounding due to temporal trend. We minimized potential influence by time via adjusting for dates since entry, and this adjustment did not affect our conclusion (data not shown). In the analysis involving the mitochondrial DNA content, we additionally adjusted for age, body mass index, and race to examine if our results are sensitive to covariates specification. Our results were robust and consistent (data not shown).

Table 2.4 Effect of medical air, B vitamins, and two-hour exposure to fine particles ($PM_{2.5}$) on mitochondrial DNA copy number, and the attenuated $PM_{2.5}$ effect due to B Vitamins treatment

	% Change*	*P*	95% CI
	Post-exposure		
Effect of medical air	-6.96	0.29	-20.00 to 6.08
Effect of B vitamins	-5.25	0.39	-17.42 to 6.93
Effect of $PM_{2.5}$, compared to medical air	1.14	0.82	-8.71 to 10.99
$PM_{2.5}$ Effect attenuation by B vitamins	-0.54	0.93	-13.46 to 12.38
	24-hour post-exposure		
Effect of medical air	2.23	0.24	-1.57 to 6.03
Effect of B vitamins	-4.25	0.43	-15.02 to 6.53
Effect of $PM_{2.5}$, compared to medical air	-8.11	0.002	-12.91 to -3.31
$PM_{2.5}$ effect attenuation by B vitamins	8.54	0.02	-1.17 to 15.92

* Represents the % change in mitochondrial DNA copy number associated with $PM_{2.5}$ exposure. Results were adjusted for chamber humidity, chamber temperature, and season (Spring/Summer/Fall/Winter).

Figure 2.4 Percent change in mitochondrial DNA (mtDNA) copy number after each exposure experiment, compared to pre-exposure status



DISCUSSION AND CONCLUSION

This study on a crossover intervention trial with controlled exposure experiments determined that two-hour exposure to concentrated ambient $PM_{2.5}$ (250μ/m^3) has substantial impact on the dynamic epigenetic landscape in circulating CD4+ T_h cells among healthy adults. Our trial demonstrated that these effects can be counteracted with B vitamins supplement (i.e., folic acid, Vitamins B_6 and B_{12}). Furthermore – as the top loci suggested potential modulation of mitochondrial function – we validated this hypothesis by showing that $PM_{2.5}$ significantly altered mitochondrial DNA content in circulating CD4+ T_h cells, and B vitamins supplement nearly completely offset these effects.

Air pollution has been consistently associated with adverse health outcomes in epidemiological studies. [46-49] In a recent study with 19 million participants for 10 follow-up years, the authors observed an 11% increase in mortality rate associated with each 10 μg/m^3 elevation in annual $PM_{2.5}$ concentrations.[5] Although the biological mechanism underlying the health effects of $PM_{2.5}$ remains unclear, several biological pathways have been proposed, including systemic inflammation and oxidative stress.[50-53] Observational studies have repeatedly shown that $PM_{2.5}$ disturbs human DNA methylation profiles,[12-14, 18, 54, 55] which might acerbate the oxidative and inflammatory responses following exposure. [12, 57, 58] Previous human exposure study showed that the *Toll-like Receptor 4* (*TLR4*) gene hypomethylation in leukocytes mediates a part of the effect of particulate air exposure on blood pressure elevation.[56] However, its candidate-gene approach limited the capability to search for novel epigenetic markers that change upon $PM_{2.5}$ exposure. Recently, an epigenome-wide association study (EWAS) examined the impact of low-concentration air pollution on DNA methylation in whole blood.[11] However, the interpretation of

their findings is limited due to potential bias due to cell-type distribution.[17] Taken together, the present study – a cell-type-specific EWAS using controlled exposure experiments – has unique advantage to provide unbiased insight on the novel epigenetic underpinnings of the pro-inflammatory and pro-oxidative effects of $PM_{2.5}$.[57]

In line with our hypothesis, the present study demonstrated the acute effects of $PM_{2.5}$ inhalation on the methylation status in the promoter region of genes that are critical in mitochondrial function and oxidative metabolism:[43, 44] *CPO*, a member of the metalloenzyme family,[44] is involved in metal ion binding, metallopeptidase, and metallocarboxypeptidase activities – which are essential in regulation of the steady-state concentration of O_2^- in the intermembrane space of mitochondria;[58] *NDUFS7* encodes one of the subunits of the mitochondrial respiratory chain complex I that transfers electrons from NADH to coenzyme Q, and *NDUFS7* mutations were of etiological significance in mitochondrial complex I deficiency.[59] Although mitochondria have their own genetic material distinct from the nuclear DNA, the majority of mitochondrial proteins are encoded by the nuclear genome.[60, 61] The observation that $PM_{2.5}$ substantially altered the methylation status of mitochondria-relevant nuclear genes indicates that mitochondria – the specialized organelles that regulate cellular-redox-balance and supplies energy – might be the immediate target of PM-induced cellular damage.[62, 63]

Our results on mitochondrial DNA content supported the findings from epigenome-wide DNA methylation scan. Exposure to $PM_{2.5}$ for two hours is followed with 8.11% (95%CI: 3.31%, 12.91%; P=0.002) reduction in mitochondrial DNA content at 24-hour post exposure, compared to medical air (Table 2.4). Mitochondria, the cytoplasmic organelles that play a pivotal role in

cellular metabolic functions and energy production, are the main immediate target of PM-induced oxidative stress.[62-64] The cellular mitochondrial genomic content is stringently regulated by biogenesis/degradation machinery,[65] which is vital in the determination of cell survival and function. To certain extent, intracellular reactive oxygen species (ROS) challenge can be buffered by compensatory mitochondria biogenesis – an adaptive stress response following environmental challenge to eliminate cellular oxidative damage.[38, 39] However, persistent oxidative stress may eventually overwhelm the adaptive response system and lead to mitochondrial DNA depletion via mitophagy.[38, 66] Our results support this hypothesis by demonstrating that exposure to high concentration $PM_{2.5}$ can reduce the mitochondrial DNA consents in circulating CD4+ T_h cells. Consistent with our results, a recent study reported that a 10 $\mu g/m^3$ increase in coarse PM (PM_{10}) exposure during pregnancy was associated with a 16.1% decrease in placental mitochondrial DNA content.[67] The present study showed that $PM_{2.5}$ exposure altered the DNA methylation status of important mitochondria-related genes immediately post exposure, and subsequently reduced the mitochondrial DNA content at 24-hour post exposure. Although formal mediation analysis was not performed in the present study due to the lack of statistical power, our result suggests that short-term $PM_{2.5}$ exposure depletes mitochondrial DNA content likely by modulating DNA methylation profile. These findings have paved the way for targeted epigenetic interventions.

DNA methylation is a modifiable biochemical process relying on methyl-group supplying nutrients such as B vitamins, and higher B vitamins intakes is postulated to increase DNA methylation levels.[10, 20, 21] This feature renders B vitamins supplement an attractive pharmaceutical intervention to counteract the effects of PM pollution, which has been associated

with loss of DNA methylation on inflammatory genes.[56] Classic experiments on the Agouti A^{vy} mice and other models have shown that dietary methyl nutrients, added during gestation[20, 24] or later even in adult life,[25-27] can be used to modulate DNA methylation status. In human studies, intake of a folic acid-depleted diet for several weeks led to hypomethylation of lymphocyte genomic DNA among post-menopausal women, and this hypomethylation was reversed when folic acid intake was subsequently increased.[28] In addition, among patients with colorectal adenomatous polyps, folic acid supplementation led to a 31% increase in leukocyte DNA methylation and a 25% increase in DNA from the colonic mucosa.[68] Potential for human translation is also demonstrated in *in vivo* studies, which showed that methyl-group supplying nutrients can be used to reverse the loss of DNA methylation induced by environmental pollutants.[29, 69] However, whether B vitamins can be used to limit adverse effects from an environmental exposure has never been tested in human studies. For the first time, our research provided the experimental evidence showing that the epigenetic and oxidative effects of $PM_{2.5}$ can be curbed using four-week B vitamins supplement. Remarkably, in our data, B vitamins supplement not only curbed PM-induced hypomethylation, but also attenuated PM-induced hypermethylation. These findings suggest that B vitamins have beneficial effects on the epigenome that are not restricted to acting solely as methyl group supplying nutrients. Rather, B vitamins might be a dynamic regulator that can both up-regulate and down-regulate the methylation status.

A major innovation of the present study over previous human epigenetics studies is the use of isolated CD4+ T_h cells for epigenome-wide DNA methylation analysis. The Houseman cell proportion estimates indicated high purity of the analyzed samples, therefore the associations

observed between $PM_{2.5}$ or B vitamins and DNA methylation is unlikely due to changes in leukocyte composition. Considering the crossover design of the present study, we arranged samples from the same volunteer on the same chip. We ensured that the exposure and treatment status of the volunteer are independent of the plating scheme by randomizing samples from the same study subjects across rows to minimize bias due to technical variables. The pre- and post-exposure samples were plated on the same row to minimize row effect. Thus, the measurement error of DNA methylation can be assumed to be nondifferential and therefore likely to bias the results towards the null hypothesis. To rule out that our results reflect false-positive findings, we conducted permutation test, and further validated the EWAS results with a widely accepted mitochondrial marker – mitochondrial DNA content – with a highly reproducible RT-PCR method. The crossover design of the present study controlled for time invariant factors such as gender, race, body mass index, etc. In addition, all exposure experiments were conducted at the same time of the day to eliminate impact due to diurnal variation.

We acknowledge several limitations in the present study. Our EWAS is underpowered to meet the Bonferroni threshold for significance with only 10 volunteers (30 exposure experiments). We selected the top loci based on both effect size and statistical significance, because those loci are more likely to infer biological significance. We could not randomize on the treatment (placebo *vs* B vitamins) order due to long biological half-life of body stores of B vitamins,[45] which created potential confounding due to temporal trend or learning effect (i.e., the volunteers might be more tolerant with the PM effects at the second PM exposure). The short study duration with only four-week time intervals also reduced the impact of temporal trend. In the sensitivity analysis, we adjusted for the amount of time spent since the study entry, and our results were robust and

consistent. While residual confounding is possible, considering the magnitude of our effect estimates and the consistency of our findings, it is unlikely that the observed association reflected bias resulting from confounding.

The unclear mechanistic underpinning of $PM_{2.5}$'s health effects remains the major gap in current knowledge – therefore creating enormous challenges to develop preventative strategies. The present study is the first human intervention trial in the investigation of novel mechanistic pathway linking air pollution and adverse health effects, and potential targeted preventive approaches. We demonstrated that ambient $PM_{2.5}$ exposure peak has unfavorable epigenetic and pro-oxidative effects, which, can be neutralized by B vitamins supplementation. Our findings suggest promising opportunities to aid the development of novel intervention strategies – which is particularly important for pathologies related to non-preventable exposures such as $PM_{2.5}$ pollution. Future trials with a larger sample size are warranted to shed light on the precise pathophysiological processes of PM-induced inflammatory and oxidative responses, as well as the mechanism underlying the protective effect of B vitamins.

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Chapter 3

Cardiac Autonomic Dysfunction: Particulate Air Pollution Effects are Modulated by Epigenetic Immunoregulation of *Toll-like Receptor 2* and Dietary Flavonoid Intake

Background: Short-term fine particles ($PM_{2.5}$) exposure is associated with reduced heart rate variability (HRV), a strong predictor of cardiac mortality among older people. Identifying modifiable factors that confer susceptibility is essential for intervention. We evaluated whether *Toll-like Receptor 2 (TLR2)* methylation, a reversible immune-epigenetic process, and its dietary modulation by flavonoids and methyl nutrients, modify susceptibility to HRV effects following $PM_{2.5}$ exposure.

Methods and Results: We measured HRV and $PM_{2.5}$ repeatedly over 11 years (1,275 total observations) among 573 elderly men from the Normative Aging Study. Blood *TLR2* methylation was analyzed using pyrosequencing. Daily flavonoid and methyl nutrients intakes were assessed through the Food Frequency Questionnaire. Every 10 $\mu g/m^3$ increase in 48-hour $PM_{2.5}$ moving average was associated with 7.74% (95%CI: -1.21%, 15.90%; P=0.09), 7.46% (95%CI: 0.99%, 13.50%; P=0.02), 14.18% (95%CI, 1.14%, 25.49%; P=0.03), and 12.94% (95%CI, -2.36%, 25.96%; P=0.09) reductions in root mean square of successive differences (rMSSD), standard deviation of normal-to-normal intervals (SDNN), low-frequency (LF) power, and high-frequency (HF) power, respectively. Higher *TLR2* methylation exacerbated the rMSSD, SDNN, LF, and HF reductions associated with heightened $PM_{2.5}$ ($P_{interaction}$=0.006, 0.03, 0.05, 0.04, respectively). Every interquartile-range increase in flavonoid intake was associated with 5.09% reduction in mean *TLR2* methylation (95%CI, 0.12%, 10.06%; P=0.05) and counteracted the effects of $PM_{2.5}$ on LF ($P_{interaction}$=0.05). No significant effect of methyl nutrients on *TLR2* methylation was observed.

Conclusions: Higher *TLR2* methylation may confer susceptibility to adverse cardiac autonomic effects of $PM_{2.5}$ exposure in older individuals. Higher flavonoid intake may attenuate these effects, possibly by decreasing *TLR2* methylation.

INTRODUCTION

Particulate matter (PM) exposure contributes to an estimated 3.7 million annual deaths worldwide.[1] According to the American Heart Association (AHA), PM exposure is a modifiable primary contributor to cardiovascular morbidity and mortality, often resulting from acute cardiovascular effects after short-term exposure peaks.[2] The major sources of $PM_{2.5}$ pollution are combustion activities (motor vehicles, power generation, and industrial processes), biomass burning, and other human activities such as heating and cooking.[2] Because prevention of exposure to PM is particularly challenging—more than 80% of people in the United States (US) receive some exposure—it is essential to identify modifiable factors that contribute to individual susceptibility to cardiac effects to aid the development of effective interventions.

Short-term PM exposure has been associated with reduced heart rate variability (HRV) through epidemiological studies.[3,4] Impaired autonomic modulation of the rhythmic activity of the sinus node, as reflected in reduced HRV, represents a pathophysiologic mechanism by which air pollution may lead to cardiac mortality, especially among older individuals.[5,6] Notably, HRV shows wide inter-individual variability, as well as highly variable responses to PM exposure.[7] Therefore, HRV is an ideal early surrogate marker of cardiovascular autonomic dysfunction for identifying underlying factors that may modify susceptibility to cardiovascular effects of PM exposure. Further, growing evidence indicates that systemic inflammation exacerbates HRV disturbances following PM exposure.[8] PM with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) is especially deleterious because it penetrates into the alveoli and may act as a stimulus to trigger local cytokine production and systemic inflammation.[9] Thus, immunoregulation is critical to limiting immune-mediated cardiovascular pathology.[10]

Molecules involved in immunoresponses are attractive as potential modulators of cardiovascular pathology following PM exposure. Toll-like receptors (TLRs), a group of receptors abundantly expressed on leukocytes, have emerged as crucial first-responders linking innate and adaptive immunity after environmental challenge.[11] TLR2, in particular, is a unique TLR family member that not only assists the clearance of bacterial components contained in PM via pathogen recognition,[12] but also modulates the expansion and behavior of regulatory T cells (Tregs)—the dominant circulating regulator of immunosuppression.[13,14] *TLR2*-related immunity is controlled through epigenetic mechanisms: increased methylation in the *TLR2* promoter region is usually associated with *TLR2* silencing, while decreased methylation permits *TLR2* expression.[15] Therefore, maintaining a proper methylation level of the *TLR2* gene is critical to ensure protective immunity.

Importantly, DNA methylation is reversible, thereby providing unique opportunities for modulation. This feature is critical, particularly for pathologies related to non-preventable exposures, as it can offer potential interventions to ameliorate exposure-related disease. Nutrients such as flavonoids and methyl nutrients can modify gene-specific methylation in opposite directions.[16] Flavonoids, the most common group of polyphenolic compounds in the human diet, can lower DNA methylation by reducing DNA methyltransferase activity.[17,18] Methyl nutrients such as folic acid, vitamin B12, and methionine, on the other hand, may increase methylation by providing methyl donors.[19] Thus, determining how such nutrients alter methylation states can provide insight that may be used to counteract exposure effects.

No study has yet investigated whether DNA methylation and nutrient intake may modify the cardiovascular effects of air pollution exposure. In this study, we hypothesized that programming of *TLR2* inactivation by increased methylation contributes to "epigenetic susceptibility" for immune-mediated PM-induced cardiovascular responses, as reflected in steeper reductions in HRV following short-term $PM_{2.5}$ exposure. We utilized the Normative Aging Study, a cohort of aging men in Eastern Massachusetts, to investigate the relationship between short-term $PM_{2.5}$ (48 hours) exposure and HRV, and to examine whether *TLR2* methylation in blood leukocytes modifies this relationship. We also examined the effect of flavonoid and methyl nutrients (folic acid, vitamin B12, and methionine) intakes on *TLR*2 methylation and explored their role as modifiers of the short-term effects of ambient $PM_{2.5}$ exposure on HRV. In addition to our primary hypothesis, we examined the effect of black carbon, carbon monoxide, nitrogen dioxide, and ozone on HRV, as well as the relationship between *TLR2* methylation and plasma inflammatory markers including interleukin 6 (IL6), IL8, IL1β, tumor necrosis factor alpha (TNFα), TNFγ, C-reactive protein (CRP), intercellular adhesion molecule 1 (ICAM-1), and vascular endothelial growth factor (VEGF).

METHODS

Study Population

The Normative Aging Study (NAS) is an ongoing prospective cohort of older male participants established in Eastern Massachusetts by the U.S. Veterans Administration (VA).[20] For the present study, we excluded participants with incomplete data on $PM_{2.5}$ exposure and HRV, leaving a total of 573 participants who were community-dwelling men free of heart arrhythmias at examination (Figure 3.1). Participants were recalled for visits every three to five years and we considered visits conducted since November 2000 (i.e., the earliest date for which $PM_{2.5}$ data were available) through 2011, for a total of 1,275 visits (one visit to four visits per participant; average 2.2 visits). Among the 573 participants, 500 participants had methylation data, 513 participants had flavonoid intake data, and 482 participants had methyl nutrients intake data. The study was approved by the institutional review boards of all participating institutions. All subjects provided informed consent.

HRV Measurement

HRV was measured in the morning for seven minutes with a 2-channel electrocardiography (ECG) monitor, and time-domain variability and frequency-domain variability of the heart rate were obtained as described previously.[3] Observations with HRV measurement time < 3.5 min were excluded. The root mean square of successive differences (rMSSD) and standard deviation of normal-to-normal intervals (SDNN) were measured representing the time domain.[21] Low-frequency (LF) (0.04-0.15 Hz) and high-frequency (HF) (0.15-0.4 Hz) power were used to represent the frequency domain.[21]

Blood *TLR2* Methylation and Plasma Inflammatory Markers

TLR2 methylation was analyzed on blood samples, collected after overnight fasting, at five CpG positions within the promoter region (Figure 3.2) using bisulfite-treated pyrosequencing, as described previously.[22] Exact positions and pyrosequencing primers were reported previously.[20] The degree of methylation is reported as the ratio of methylated cytosines over the sum of methylated and unmethylated cytosines (%5mC). Plasma inflammatory markers including IL6, IL8, IL1β, TNFα, TNFγ, CRP, ICAM-1, and VEGF were measured using previously described methods.[20,22,23]

Air Pollution and Weather Data

$PM_{2.5}$ exposure levels across the study area were estimated based on a validated hybrid spatio-temporal prediction model,[24] which was used in previous epidemiological studies.[25,26] This exposure assessment method combined real physical measurements from the MODIS (Moderate Resolution Imaging Spectroradiometer) satellite-derived aerosol optical depth (AOD) and classic land-use regression methods to predict daily $PM_{2.5}$ concentration levels across the Boston area from 2000 to 2011, at a 10 × 10 km spatial resolution. Kloog and coauthors demonstrated that this method provides more accurate spatial resolution and unbiased predictions compared to prior models[27] without compromising temporal resolution.[28] For more methodological details of the prediction model please refer to Kloog et al.[28,29] The 48-hour $PM_{2.5}$ moving averages before each examination were assigned based on the participants' home addresses. The 48-hour moving averages of outdoor apparent temperature was tabulated based on outdoor air temperature, relative humidity, and wind speed, which were obtained from the Boston airport weather station. Black carbon concentration was measured at the Harvard School of Public Health monitoring

site, 1 km from the exam site, using an aethalometer (Magee Scientific, Berkeley, CA).[30] Carbon monoxide, nitrogen dioxide, and ozone were obtained from the Massachusetts Department of Environmental Protection local monitoring sites, using previously descripted method.[30]

Food Frequency Questionnaire and Nutrient Intake

At every visit, detailed information on the typical daily dietary intake of food and beverage items over the previous year was assessed with a self-administered validated semi-quantitative Food Frequency Questionnaire (FFQ) adapted from the questionnaire used in the Nurses' Health Study. Details on the reproducibility and validity of this FFQ for estimating daily nutrient intakes,[31,32] including flavonoid[33] and methyl nutrients (folic acid, vitamin B12, and methionine) intake[21] were published elsewhere. To estimate flavonoid intakes, we constructed a database for assessment of intake of the different flavonoid subclasses using the updated and expanded U.S. Department of Agriculture (USDA) flavonoid content of foods and the proanthocyanidin databases together with other sources.[34,35] Intakes of individual compounds were calculated as the sum of the consumption frequency of each food multiplied by the content of the specific flavonoid for the specified portion size. For foods in the FFQ for which there were no values available in the USDA database, we searched a European database (EuroFIR eBASIS; http://www.eurofir.org) and other sources to ensure all available high-quality data on flavonoid values could be included in the database. However, although these other sources served as a validation to the USDA database, the addition of the EuroFIR data and the published literature did not contribute to more than 5–10% of the overall data used in the calculation of intakes for this analysis. For each participant and visit in this analysis, we derived average daily intakes (mg/day) at each visit of the subclasses commonly consumed in the U.S. diet. The specific

subclasses included anthocyanins (cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin), flavanones (eriodictyol, hesperetin, naringenin), flavan-3-ols (catechins, gallocatechins, epicatachin, epigallocatechin, epicatachin-3-gallate, epigallocatechin-3-gallate), flavonols (quercetin, kaempferol, myricetin, isohamnetin), flavones (luteolin, apigenin), and polymers (including proanthocyanidins excluding monomers, theaflavins, and thearubigins). The measure "total flavonoids" represented the sum of these six subclasses. Assessment of daily dietary folic acid intake was based on the frequency and dosage information from the FFQ. The average daily total fruit and total vegetable intake (servings/day) was also measured based on FFQ, over the past year. The estimates were made using software developed by the Nurses' Health Study at the Channing Laboratories and processed by Channing Laboratories operators. To avoid undue influence of outliers, we excluded all the questionnaires reporting <500 or >4200 total calories per day. We also truncated flavonoid and folic acid intakes at median ± 3×(interquartile-range). Fewer than 0.5% of observations were excluded.

Statistical Methods

Confounding Selection and Model Assumption

For the analysis involving $PM_{2.5}$ exposure, *TLR2* methylation, and HRV, we adjusted for potential confounders, selected based on literature evidence, i.e., age, room temperature, outdoor apparent temperature, season (winter/spring-fall/summer), household income, weekday of the visit, and the visit date. To increase efficiency, we also adjusted for the following risk factors for decreased HRV: body mass index (BMI), smoking status (never/former/current), physical activity, fasting glucose, alcohol consumption (< 2 drinks/day/≥ 2 drinks/day), hypertension, and use of calcium channel blockers, β-blockers, and angiotensin-converting enzyme inhibitors. For

the analysis examining flavonoid and methyl nutrients intake as effect modifier(s) for the association between $PM_{2.5}$ exposure and HRV, we adjusted for all the covariates listed above, as well as for total fiber, vitamin C, and caloric intakes. To examine the effect of flavonoid and methyl nutrients intakes on *TLR*2 methylation, we adjusted for age, BMI, smoking status, total fiber, vitamin C, caloric intake, household income, and physical activity.

HR, rMSSD, SDNN, LF, and HF were $\log_{10}$-transformed to improve normality and stabilize the variance. All independent variables were fitted as time-varying covariates. Non-linear relationships were characterized first using graphical analyses between HRV and all the covariates, as well as between nutrient intakes and *TLR*2 methylation. When the graphical analyses suggested non-linear association, higher order terms (quadratic, cubic, etc.,) were considered in the model, and a likelihood ratio test was used to determine the number of higher order terms needed. In the present study, nonlinearity between apparent temperature and HRV was accounted for by the use of linear and quadratic terms. In addition, we utilized the likelihood ratio test to determine whether it is adequate to model the effect modifiers with a linear trend. Additional analysis using spline regression models were also used to confirm linearity.

To account for repeated assessments for many participants, all statistical models were linear mixed effect models with a random intercept assigned to each subject, which used compound symmetry covariance structure. A compound symmetry covariance structure was decided upon after comparing the Akaike information criterion (AIC) among models with different covariance structures as well as assessing the fit of the compound symmetry covariance structure to our data by examining the estimated residual covariance matrix. An analysis using a robust/sandwich estimator for the variance gave very similar results and thus analysis without use of

robust/sandwich variance estimators were reported in the manuscript. To examine whether any position in the *TLR*2 promoter region modified the association between $PM_{2.5}$ exposure and HRV, we fitted a single linear mixed effect model with five interaction terms included in the model, which are the interactions between each of the five *TLR2* positions and $PM_{2.5}$ (in addition to their respective main effects and other covariates) **(Model 1)**. To test the null hypothesis that none of the positions modified the association between $PM_{2.5}$ exposure and HRV (H_0: $\gamma_1 = \gamma_2 = ... = \gamma_5 = 0$), the coefficients of these five interaction terms were tested all together using a single Wald test, whose null distribution is approximated using a F-distribution (global test).

$$Y_{ij} = \beta_0 + \beta_1 PM_{2.5ij} + \beta_2 X_{2ij} + \ldots + \beta_p X_{pij} + \alpha_1 TLR2_{1ij} + \ldots + \alpha_5 TLR2_{5ij} + \gamma_1 PM_{2.5ij} * TLR2_{1ij} + \ldots + \gamma_5 PM_{2.5} * TLR2_{5ij} + b_i + \varepsilon_{ij} \quad \text{(Model 1)}$$

As a secondary analysis, we also examined the effect modification by position-specific *TLR2* methylation by including an interaction term between position-specific *TLR2* methylation and $PM_{2.5}$ in the model **(Model 2)**, only if the global test was significant.

$$Y_{ij} = \beta_0 + \beta_1 PM_{2.5ij} + \beta_2 X_{2ij} + \ldots + \beta_p X_{pij} + \alpha_1 TLR2_{kij} + \gamma_1 PM_{2.5} * TLR2_{kij} + b_i + \varepsilon_{ij} \quad \text{(Model 2)}$$

where Y_{ij} was the $\log_{10}$ of HRV measures for participant i at visit j, β_0 was the overall intercept, b_i was the separate random intercept for subject i, and $b_i \sim N(0, \Theta)$, $\varepsilon_{ij} \sim N(0, \sigma^2)$. $TLR2_{kij}$ was the *TLR2* methylation level at the k_{th} position (k=1-5) within the promoter region, for participant i at visit j. X_{2ij}–X_{pij} were the covariates including confounders and predictors for HRV, for participant i at visit j.

To describe the effect modification by potential effect modifiers, we estimated from the mixed effect models the HRV-$PM_{2.5}$ associations at the values corresponding to the quartiles of each effect modifier and reported them separately. We reported effect estimates, 95% confidence

intervals, and *P* values for the HRV-$PM_{2.5}$ associations from the linear mixed effect models (i.e. % change in HRV per 10μg/m^3 increase in $PM_{2.5}$) for the midpoint of each quartile. Analyses were performed using SAS 9.4 (SAS Institute, Cary NC).

RESULTS

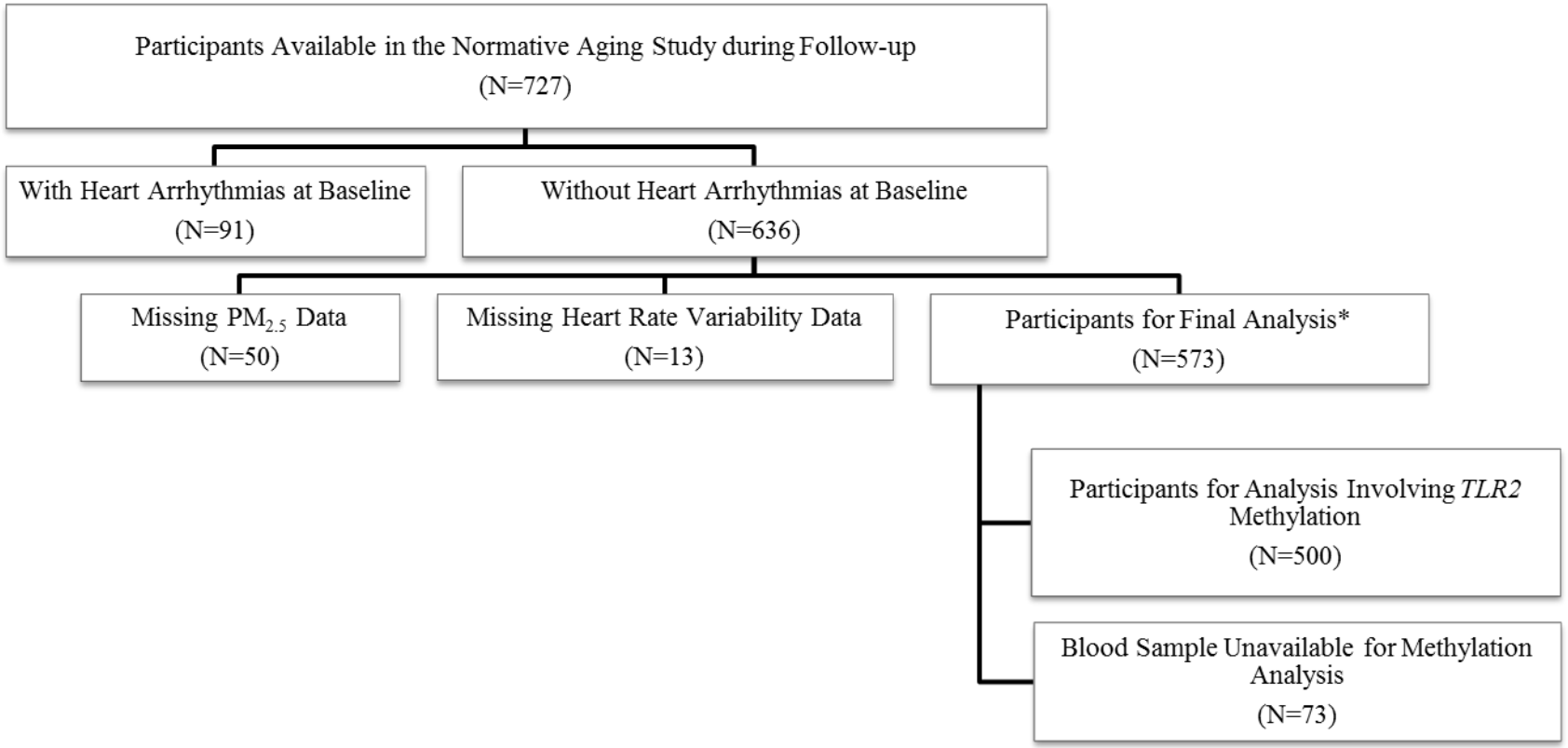
Cohort Characteristics and Exposure Levels

The NAS is a cohort of aging individuals, between 55 and 100 years old at the first visit of the present study (Figure 3.1). We excluded N=91 participants with heart arrhythmias at baseline. An additional N=63 participants were excluded as they had missing outcome (N=13 with missing HRV) or missing exposure (N=50 with missing $PM_{2.5}$ exposure), giving a total of N=573. The baseline characteristics of the participants included in the final analysis are given in Table 1. All participants were male, and 96% were white. Seventy-nine percent of the participants were overweight, 19% were diabetics, and 71% were hypertensive. The study population included 5% current smokers and 19% heavy alcohol drinkers (≥ 2 drinks/day). During the study period (November 2000-December 2011), the 48-hour $PM_{2.5}$ moving average varied between 2.14 $\mu g/m^3$ to 42.8 $\mu g/m^3$ with an average exposure level of 10.5 $\mu g/m^3$, which is lower than the U.S. Environmental Protection Agency's daily health National Ambient Air Quality Standard for $PM_{2.5}$ (35.0 $\mu g/m^3$). The baseline mean methylation level across five CpG positions (Figure 3.2) in the *TLR2* promoter region ranged between 0.3 %5mC and 7.1 %5mC. There was no apparent difference in exposure level or methylation level across different characteristic subgroups (Table 3.1).

Main Effect of Air Pollutants on HRV

Average $PM_{2.5}$ exposure over the 48 hours before the day of each visit was associated with significantly lowered SDNN and LF, marginally lowered rMSSD and HF, and non-significantly increased HR. Every 10 $\mu g/m^3$ increase in the 48-hour $PM_{2.5}$ moving average was associated with 7.74% (95% CI, -1.21%, 15.90%; *P*=0.09), 7.46% (95% CI, 0.99%, 13.50%; *P*=0.02),

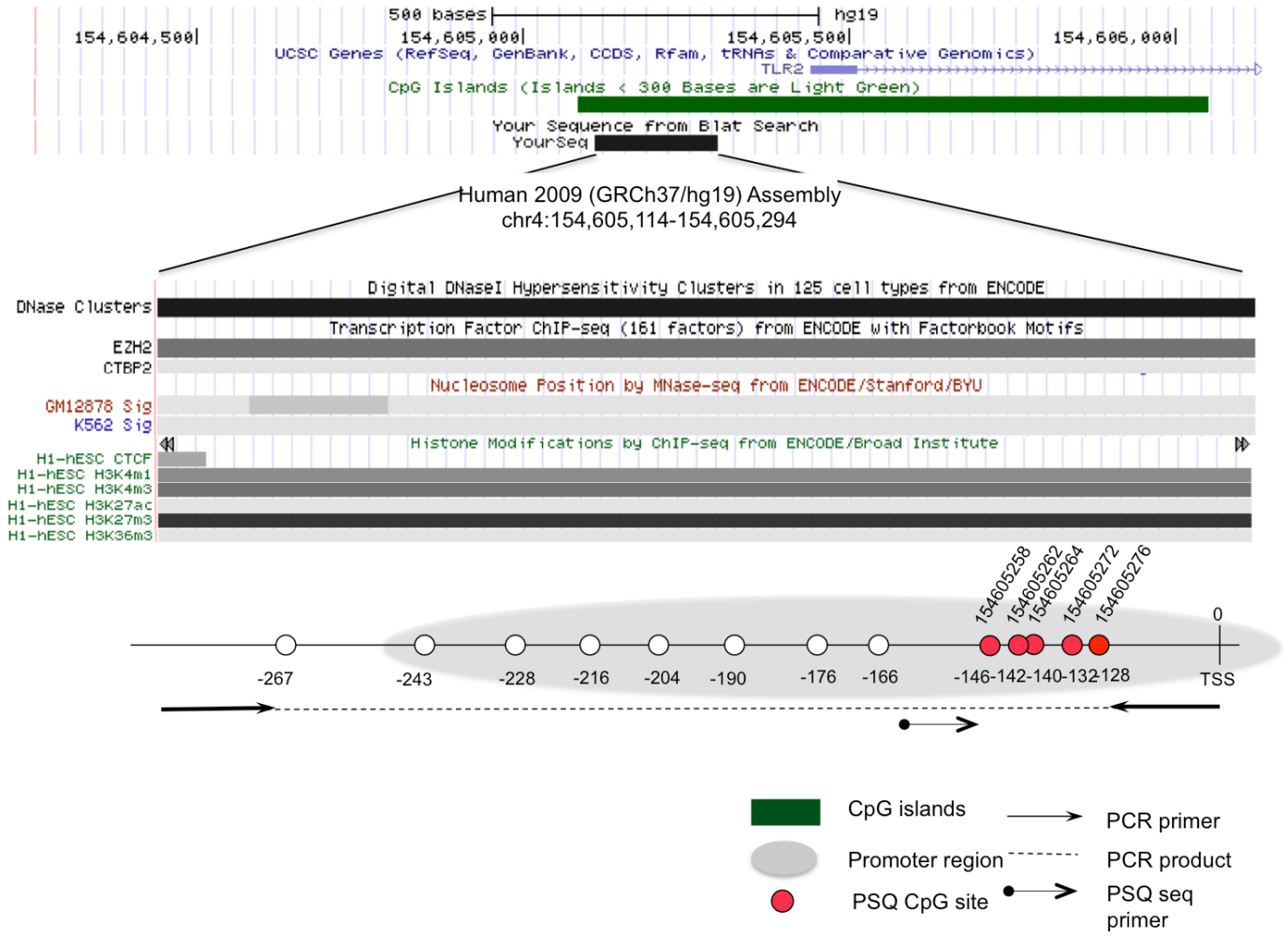
Figure 3.1. The study participants in the Normative Aging Study, 2000-2011



$PM_{2.5}$ indicates particulate matter with aerodynamic diameter < 2.5 micrometers

*In each analysis, some additional participants may have been excluded due to missing key study variables. We have noted in each table the corresponding sample size.

Figure 3.2. Schematic view of the genomic structure and CpG dinucleotides selected for analysis in the *Toll-like Receptor 2* (*TLR2*) gene



The chromosomal location of the PCR amplicon for the *TLR2* gene pyrosequencing assay is based on the Human Genome Assembly 2009 (GRC37/hg19) (UCSC Genome Browser). The CpG island, gene promoter region, PSQ CpG sites, PCR primer, and PSQ sequence primer location are shown in the figure. TSS indicates the transcription start site. Position 1: CpG154605258; Position 2: CpG154605262; Position 3: CpG154605264; Position 4: CpG154605272; Position 5: CpG154605276

Table 3.1. Baseline characteristics of study participants (N=573), 48-hour moving average of particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$), and averaged methylation across CpG positions in the promoter region of *Toll-like Receptor 2* gene

Characteristics	N (%)	$PM_{2.5}$ ($\mu g/m^3$) mean (SD)	*TLR2* methylation (%5mC) mean (SD)
Age (years)			
55-69	176 (30.7)	11.4 (5.2)	2.9 (1.3)
70-79	293 (51.1)	11.7 (6.4)	3.1 (1.3)
80-89	97 (16.9)	10.6 (6.1)	2.9 (1.1)
> 90	7 (1.2)	13.0 (5.0)	3.4 (1.0)
Physical activity (MET-hr/week)			
< 12	371 (64.8)	11.4 (6.3)	2.93 (1.2)
12 to < 30	114 (19.9)	11.3 (5.3)	3.14 (1.4)
≥ 30	88 (15.4)	11.7 (5.5)	3.08 (1.2)
Alcohol (drinks/day)			
≥ 2	106 (18.5)	11.6 (6.7)	2.9 (1.1)
< 2	467 (81.5)	11.4 (5.8)	3.0 (1.2)
Diabetes			
Yes	111 (19.4)	11.0 (6.0)	2.9 (1.0)
No	462 (80.6)	11.5 (6.0)	3.0 (1.3)
Race			
White	549 (95.8)	11.4 (5.9)	3.0 (1.3)
Non-white	24 (4.2)	11.6 (7.7)	2.8 (0.9)
Hypertension			
Yes	405 (70.7)	11.3 (6.0)	3.0 (1.3)
No	168 (29.3)	11.8 (6.0)	2.9 (1.2)
BMI (kg/m^2)			
< 25	123 (21.5)	12.4 (6.8)	3.0 (1.2)
≥ 25	450 (78.5)	11.2 (5.7)	3.0 (1.3)
Smoking			
Never	166 (29.0)	11.7 (6.0)	3.1 (1.4)
Current	26 (4.5)	10.9 (3.8)	3.3 (1.1)
Former	381 (66.5)	11.4 (6.1)	2.9 (1.2)
Annual Income > $60,000			
Yes	272 (47.5)	11.0 (5.4)	3.1 (1.3)
No	301 (52.5)	11.8 (6.5)	2.9 (1.2)

MET indicates metabolic equivalent of task; BMI indicates body mass index

14.18% (95% CI, 1.14%, 25.49%; *P*=0.03), and 12.94% (95% CI, -2.36%, 25.96%; *P*=0.09) reductions in rMSSD, SDNN, LF, and HF, respectively (Table 3.2). Exposure to black carbon, carbon monoxide, nitrogen dioxide, and ozone over the 48 hours before the day of each visit were not significantly associated with HR, rMSSD, SDNN, LF, and HF (Table 3.3).

Modification of $PM_{2.5}$ Effect on HRV by *TLR2* Methylation

The effect of 48-hour $PM_{2.5}$ exposure at participants' homes on rMSSD, SDNN, LF, and HF was significantly modified by the level of methylation within the *TLR2* promoter region (*P*=0.006 for rMSSD; *P*=0.03 for SDNN; *P*=0.05 for LF, and *P*=0.04 for HF, global test for interaction across five positions) (Table 3.4). $PM_{2.5}$ exposure had no significant effect on HRV in individuals with low *TLR2* methylation, but it showed increasingly negative effects on HRV as *TLR2* methylation increased (Figure 3.3). For instance, in individuals with *TLR2* methylation within the first quartile, every 10 $\mu g/m^3$ increase in 48-hour $PM_{2.5}$ exposure was associated with 8.77% (95% CI, -7.55%, 27.95%; *P*=0.31), -0.39% (95% CI, -11.76%, 12.45%; *P*=0.95), -7.25% (95% CI, -27.74%, 19.06%; *P*=0.56), and 10.06% (95% CI, -16.25%, 44.64%; *P*=0.49) non-significant estimated changes in rMSSD, SDNN, LF, and HF, respectively (effects estimated at the mid-point of the 1st quartile, i.e., 1.5 %5mC to represent the within-quartile effect, see Table 3.4); on the other hand, in the fourth *TLR2* methylation quartile, every 10 $\mu g/m^3$ increase in 48-hour $PM_{2.5}$ exposure was associated with 26.38% (95% CI, 13.69%, 37.20%; *P*=0.0002), 13.94% (95% CI, 3.12%, 23.55%; *P*=0.01), 21.40% (95% CI, -0.39%, 38.47%; *P*=0.05), and 34.28% (95% CI, 13.84%, 49.87%; *P*=0.003) reductions in rMSSD, SDNN, LF, and HF, respectively (effects estimated at the mid-point of the 4th quartile, i.e., 4.4 %5mC to represent the within-quartile effect). Analysis of individual position-specific methylation showed notable effect

Table 3.2. Effect of 48-hour moving average of particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) on heart rate variability (HRV), Normative Aging Study, 2000-2011 (N=500)

	Change in HRV per 10 μg/m^3 increase in $PM_{2.5}$ concentration*		
HRV measure	% Change	95% CI	*P*
HR	0.42	-0.94 to 1.80	0.55
rMSSD	-7.74	-15.90 to 1.21	0.09
SDNN	-7.46	-13.50 to -0.99	0.02
LF	-14.18	-25.49 to -1.14	0.03
HF	-12.94	-25.96 to 2.36	0.09

HR indicates heart rate; rMSSD indicates the root mean square of successive differences; SDNN indicates the standard deviation of normal-to-normal intervals; LF indicates low-frequency power (0.04–0.15 Hz); HF indicates high-frequency power (0.15–0.4 Hz); CI indicates confidence interval.

*Results were adjusted for age; body mass index; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise; household income; the use of calcium channel blocker, β-blocker, and angiotensin-converting enzyme inhibitor; room temperature; outdoor apparent temperature; season; weekday; and visit date.

Table 3.3. Effect of 48-hour moving average of black carbon, carbon monoxide, nitrogen dioxide, and ozone on heart rate variability (HRV), Normative Aging Study, 2000-2011 (N=573)

	Black Carbon			Carbon Monoxide			Nitrogen Dioxide			Ozone		
HRV	% Change*	95% CI	*P*	% Change*	95% CI	*P*	% Change*	95% CI	*P*	% Change*	95% CI	*P*
HR	0.04	-0.82 to 0.92	0.92	-0.97	-3.16 to 1.28	0.40	0.25	-0.76 to 1.27	0.63	-0.92	-2.19 to 0.37	0.16
rMSSD	-2.02	-7.59 to 3.88	0.49	-1.86	-15.63 to 14.15	0.81	-3.71	-10.08 to 3.11	0.28	-5.31	-13.2 to 3.3	0.22
SDNN	-2.28	-6.36 to 1.98	0.29	-2.19	-12.42 to 9.23	0.69	-1.93	-6.72 to 3.1	0.44	-4.13	-10.04 to 2.17	0.19
LF	-2.46	-10.82 to 6.68	0.59	-1.61	-21.91 to 23.96	0.89	-1.55	-11.31 to 9.3	0.77	-5.92	-17.66 to 7.5	0.37
HF	-1.91	-11.45 to 8.67	0.71	-2.55	-25.13 to 26.86	0.85	-7.29	-17.7 to 4.44	0.21	-5.19	-18.57 to 10.39	0.49

HR indicates heart rate; rMSSD indicates the root mean square of successive differences; SDNN indicates the standard deviation of normal-to-normal intervals; LF indicates low-frequency power (0.04–0.15 Hz); HF indicates high-frequency power (0.15–0.4 Hz); CI indicates confidence interval.

*Results were adjusted for age; body mass index; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise; household income; the use of calcium channel blocker, β-blocker, and angiotensin-converting enzyme inhibitor; room temperature; outdoor apparent temperature; season; weekday; and visit date.

Table 3.4. The effect of particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) exposure on heart rate variability (HRV) at different mean *Toll-like Receptor 2* (*TLR2*) methylation levels, Normative Aging Study, 2000-2011 (N=500)

	HR		
Mean *TLR2* Methylation	% Change*	95% CI	*P*
Midpoint of Q1 (1.5 5mc%)	-0.56	-2.88 to 1.82	0.64
Midpoint of Q2 (2.2 5mc%)	-0.10	-1.99 to 1.81	0.92
Midpoint of Q3 (3.0 5mc%)	0.42	-1.25 to 2.11	0.62
Midpoint of Q4 (4.4 5mc%)	1.34	-0.92 to 3.66	0.25
P_{global} for Interaction†			0.62
	rMSSD		
Mean *TLR2* Methylation	% Change*	95% CI	*P*
Midpoint of Q1 (1.5 5mc%)	8.77	-7.55 to 27.95	0.31
Midpoint of Q2 (2.2 5mc%)	-1.01	-13.14 to 12.81	0.88
Midpoint of Q3 (3.0 5mc%)	-11.11	-20.87 to -0.16	0.05
Midpoint of Q4 (4.4 5mc%)	-26.38	-37.20 to -13.69	0.0002
P_{global} for Interaction†			0.006
	SDNN		
Mean *TLR2* Methylation	% Change*	95% CI	*P*
Midpoint of Q1 (1.5 5mc%)	-0.39	-11.76 to 12.45	0.95
Midpoint of Q2 (2.2 5mc%)	-3.85	-12.78 to 6.00	0.43
Midpoint of Q3 (3.0 5mc%)	-7.65	-15.31 to 0.71	0.07
Midpoint of Q4 (4.4 5mc%)	-13.94	-23.55 to -3.12	0.01
P_{global} for Interaction†			0.03
	LF		
Mean *TLR2* Methylation	% Change*	95% CI	*P*
Midpoint of Q1 (1.5 5mc%)	-7.25	-27.74 to 19.06	0.56
Midpoint of Q2 (2.2 5mc%)	-10.88	-27.11 to 8.96	0.26
Midpoint of Q3 (3.0 5mc%)	-14.86	-28.80 to 1.81	0.08
Midpoint of Q4 (4.4 5mc%)	-21.40	-38.47 to 0.39	0.05
P_{global} for Interaction†			0.05
	HF		
Mean *TLR2* Methylation	% Change*	95% CI	*P*
Midpoint of Q1 (1.5 5mc%)	10.06	-16.25 to 44.64	0.49
Midpoint of Q2 (2.2 5mc%)	-2.82	-22.02 to 21.10	0.80
Midpoint of Q3 (3.0 5mc%)	-15.71	-30.75 to 2.60	0.09
Midpoint of Q4 (4.4 5mc%)	-34.28	-49.87 to -13.84	0.003
P_{global} for Interaction†			0.04

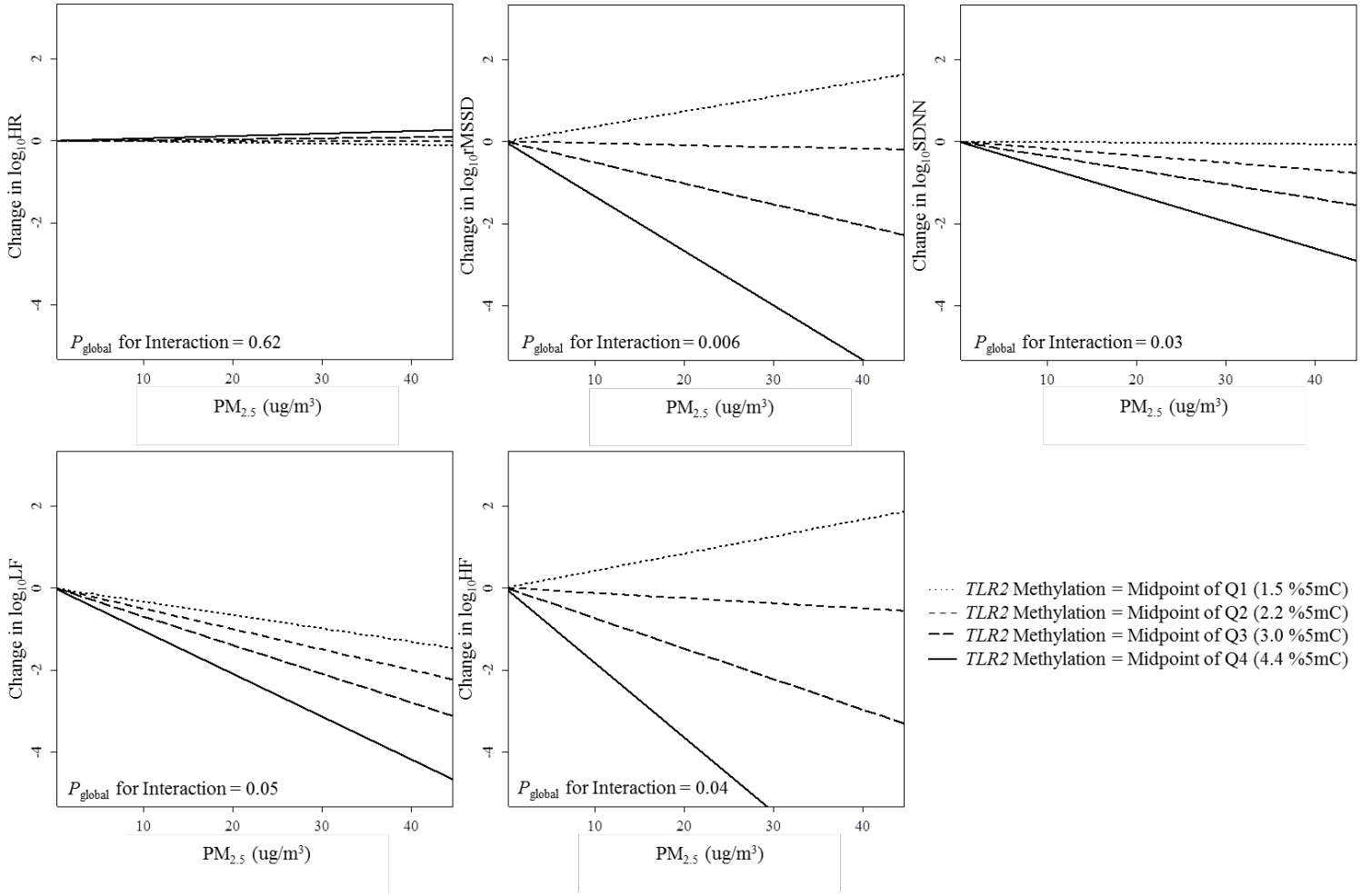
HR indicates heart rate; rMSSD indicates the root mean square of successive differences; SDNN indicates the standard deviation of normal-to-normal intervals; LF indicates low-frequency power (0.04 –0.15 Hz); HF indicates high-frequency power (0.15–0.4 Hz); CI indicates confidence interval; Q1, Q2, Q3, and Q4 indicate the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} quartile.

Results were adjusted for age; body mass index; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise; household income; the use of calcium channel blocker, β-blocker, and angiotensin-converting enzyme inhibitor; room temperature; outdoor apparent temperature; season; weekday; and visit date.

*The percent change in HRV per 10 $\mu g/m^3$ increase in $PM_{2.5}$ concentration.

†Based on a global test for effect modification by position-specific methylation. Interaction terms between each of the five *TLR2* positions and $PM_{2.5}$ were included in the model, and these five interaction terms were tested jointly for effect modification using a Wald test (global test).

Figure 3.3. The effect of particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) exposure on heart rate variability (HRV) at different mean *Toll-like Receptor 2* (*TLR2*) methylation levels, Normative Aging Study, 2000-2011 (N=500)



log_{10}HR indicates log_{10}-transformed heart rate; log_{10}rMSSD indicates log_{10}-transformed root mean square of the successive differences; log_{10}SDNN indicates log_{10}-transformed standard deviation of normal-to-normal intervals; log_{10}LF indicates log_{10}-transformed low-frequency power (0.04 –0.15 Hz); log_{10}HF indicates log_{10}-transformed high-frequency power (0.15–0.4 Hz); Q1, Q2, Q3, and Q4 indicate the 1st, 2nd, 3rd, and 4th quartiles.

The association of $PM_{2.5}$ with rMSSD, SDNN, LF, and HF is modified by mean *TLR2* methylation levels, as indicated by the different slopes. The four lines in each figure represent the relationship between $PM_{2.5}$ and HRV when the mean *TLR2* methylation level is at the midpoints of each quartile. If there was no effect modification, the four lines would be the same. The P_{global} for Interaction was based on a global test for effect modification by position-specific methylation. Interaction terms between each of the five *TLR2* positions and $PM_{2.5}$ were included in the model, and these five interaction terms were tested jointly for effect modification using a Wald test (global test). Results were adjusted for age; body mass index; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise, household income; the use of calcium channel blocker, β-blocker, and angiotensin-converting enzyme inhibitor; room temperature; outdoor apparent temperature; season; weekday; and visit date.

heterogeneity across the five CpG positions, with stronger effect modifications by positions 1, 3, and 5 (Table 3.5).

Association of Flavonoid and Methyl Nutrients Intakes with *TLR*2 methylation

The average daily flavonoid intake over the preceding year was negatively associated with mean *TLR2* methylation, considered as the average methylation level across five CpG positions (5.09% reduction; 95% CI, 0.12%, 10.06%; per an interquartile-range increase in flavonoid intake; *P*=0.05) (Figure 3.4). In position-specific analyses (Figure 3.4 and Table 3.6), methylation at *TLR2* promoter positions 1 and 4 showed the strongest associations with flavonoid intake. We did not observe significant association between methyl nutrients (folic acid, vitamin B12, and methionine) intakes in the previous year and either mean or position-specific *TLR2* methylation (Figure 3.4 and Figure 3.5).

Effect Modification by Flavonoid and Methyl Nutrients intakes

Higher average daily flavonoid intake over the preceding year significantly weakened the association between $PM_{2.5}$ exposure and LF reduction (*P*=0.05), and produced a non-significant attenuation of the effect of $PM_{2.5}$ exposure on rMSSD, SDNN and HF (*P*=0.79 for rMSSD; *P*=0.37 for SDNN; *P*=0.28 for HF) (Table 3.7 and Figure 3.6). $PM_{2.5}$ exposure had no significant effect on LF in individuals with high flavonoid intake, but it was associated with increasingly negative effects on LF as flavonoid intakes decreased (Figure 3.6). In particular, in individuals at the first quartile of flavonoid intake, every 10 $\mu g/m^3$ increase in 48-hour $PM_{2.5}$ exposure was associated with a 16.48% (95% CI, -1.61%, 31.35%; *P*=0.07) reduction in LF (effects estimated at the mid-point of the 1st quartile, i.e., 128 mg/d to represent the within-quartile effect), while no

Table 3.5. Effect modification by position-specific *Toll-like Receptor 2* (*TLR2*) methylation on the association between particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) exposure and heart rate variability (HRV) (N=500)

Position	Methylation	HR			rMSSD			SDNN		
		% Change*	95% CI	*P*	% Change*	95% CI	*P*	% Change*	95% CI	*P*
1	Midpoint of Q1	0.36	-1.96 to 2.75	0.76	7.26	-8.65 to 25.94	0.39	1.66	-9.79 to 14.56	0.79
	Midpoint of Q2	0.42	-1.38 to 2.24	0.65	-4.56	-15.66 to 8.01	0.46	-4.30	-12.71 to 4.93	0.35
	Midpoint of Q3	0.46	-1.22 to 2.17	0.60	-13.25	-22.84 to -2.47	0.02	-8.91	-16.51 to -0.63	0.04
	Midpoint of Q4	0.53	-1.72 to 2.83	0.65	-26.01	-36.84 to -13.31	0.0002	-16.11	-25.42 to -5.64	0.004
	P for Interaction†			0.92			0.0009			0.02
2	Midpoint of Q1	0.08	-2.12 to 2.34	0.94	-0.21	-14.47 to 16.43	0.98	-7.00	-17.08 to 4.3	0.22
	Midpoint of Q2	0.28	-1.49 to 2.09	0.76	-6.61	-17.49 to 5.72	0.28	-7.36	-15.51 to 1.58	0.10
	Midpoint of Q3	0.44	-1.23 to 2.13	0.61	-11.33	-21.1 to -0.35	0.04	-7.64	-15.31 to 0.73	0.07
	Midpoint of Q4	0.75	-1.4 to 2.95	0.50	-20.06	-31.31 to -6.98	0.004	-8.20	-17.98 to 2.74	0.14
	P for Interaction†			0.65			0.03			0.86
3	Midpoint of Q1	-0.82	-3.19 to 1.61	0.51	5.96	-10.3 to 25.17	0.50	1.15	-10.63 to 14.49	0.86
	Midpoint of Q2	-0.16	-1.99 to 1.71	0.86	-3.69	-15.24 to 9.44	0.56	-3.72	-12.44 to 5.87	0.43
	Midpoint of Q3	0.37	-1.3 to 2.07	0.67	-10.77	-20.6 to 0.28	0.06	-7.44	-15.13 to 0.94	0.08
	Midpoint of Q4	1.27	-0.85 to 3.44	0.24	-21.64	-32.48 to -9.05	0.00	-13.45	-22.51 to -3.33	0.01
	P for Interaction†			0.18			0.005			0.05
4	Midpoint of Q1	-0.28	-2.62 to 2.12	0.82	2.48	-12.89 to 20.56	0.77	-4.43	-15.3 to 7.83	0.46
	Midpoint of Q2	0.11	-1.73 to 1.99	0.90	-4.88	-16.33 to 8.13	0.44	-6.13	-14.66 to 3.25	0.19
	Midpoint of Q3	0.45	-1.22 to 2.15	0.60	-10.81	-20.64 to 0.23	0.06	-7.57	-15.25 to 0.8	0.08
	Midpoint of Q4	1.10	-1.08 to 3.32	0.33	-21.06	-32.16 to -8.15	0.002	-10.25	-19.8 to 0.45	0.06
	P for Interaction†			0.38			0.01			0.43
5	Midpoint of Q1	-1.24	-3.63 to 1.2	0.32	6.37	-9.99 to 25.69	0.47	1.72	-10.17 to 15.19	0.79
	Midpoint of Q2	-0.31	-2.15 to 1.57	0.75	-3.71	-15.29 to 9.45	0.56	-3.62	-12.39 to 6.02	0.45
	Midpoint of Q3	0.48	-1.2 to 2.18	0.58	-11.31	-21.05 to -0.36	0.04	-7.82	-15.46 to 0.52	0.07
	Midpoint of Q4	1.81	-0.34 to 4	0.10	-22.79	-33.47 to -10.39	0.0007	-14.49	-23.45 to -4.47	0.006
	P for Interaction†			0.05			0.003			0.03

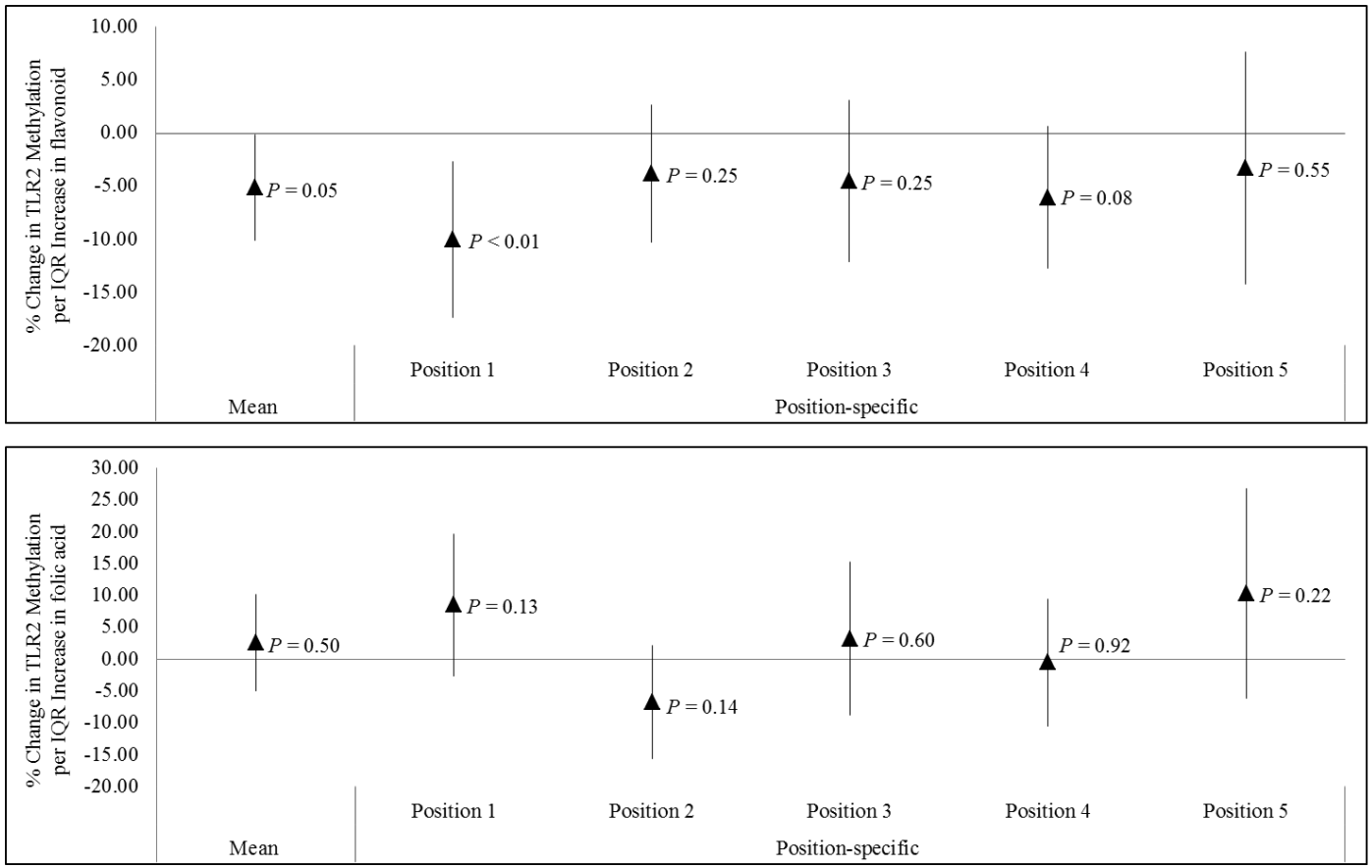
Position	Methylation	LF			HF		
		% Change*	95% CI	*P*	% Change*	95% CI	*P*
1	Midpoint of Q1	-11.35	-30.67 to 13.36	0.34	1.00	-22.87 to 32.27	0.94
	Midpoint of Q2	-13.74	-28.64 to 4.28	0.13	-9.74	-26.73 to 11.19	0.34
	Midpoint of Q3	-15.64	-29.53 to 0.98	0.06	-17.68	-32.53 to 0.45	0.06
	Midpoint of Q4	-18.73	-36.26 to 3.64	0.10	-29.38	-46.1 to -7.48	0.01
	P for Interaction†			0.61			0.06
2	Midpoint of Q1	-17.70	-34.99 to 4.19	0.11	-5.94	-27.43 to 21.91	0.64
	Midpoint of Q2	-16.04	-30.54 to 1.48	0.07	-11.67	-28.3 to 8.83	0.24
	Midpoint of Q3	-14.72	-28.69 to 1.99	0.08	-15.90	-30.99 to 2.48	0.09
	Midpoint of Q4	-12.02	-30.33 to 11.11	0.28	-23.77	-41.18 to -1.22	0.04
	P for Interaction†			0.67			0.22
3	Midpoint of Q1	4.93	-18.73 to 35.46	0.71	10.57	-16.54 to 46.49	0.48
	Midpoint of Q2	-6.44	-23.08 to 13.79	0.51	-4.44	-22.97 to 18.55	0.68
	Midpoint of Q3	-14.64	-28.62 to 2.06	0.08	-14.96	-30.18 to 3.58	0.11
	Midpoint of Q4	-26.97	-41.87 to -8.26	0.007	-30.26	-45.83 to -10.2	0.005
	P for Interaction†			0.03			0.01
4	Midpoint of Q1	-12.69	-31.92 to 11.98	0.29	-0.15	-24.04 to 31.25	0.99
	Midpoint of Q2	-13.86	-29.23 to 4.84	0.14	-8.49	-26.31 to 13.62	0.42
	Midpoint of Q3	-14.86	-28.79 to 1.8	0.08	-15.14	-30.32 to 3.36	0.10
	Midpoint of Q4	-16.72	-33.93 to 4.96	0.12	-26.43	-42.95 to -5.13	0.02
	P for Interaction†			0.77			0.09
5	Midpoint of Q1	-1.26	-23.57 to 27.57	0.92	15.29	-12.68 to 52.22	0.32
	Midpoint of Q2	-9.27	-25.47 to 10.46	0.33	-3.32	-21.96 to 19.78	0.76
	Midpoint of Q3	-15.39	-29.24 to 1.17	0.07	-16.41	-31.27 to 1.68	0.07
	Midpoint of Q4	-24.79	-40.16 to -5.46	0.01	-34.57	-49.1 to -15.88	0.001
	P for Interaction†			0.10			0.002

HR indicates heart rate; rMSSD indicates the root mean square of successive differences; SDNN indicates the standard deviation of normal-to-normal intervals; LF indicates low-frequency power (0.04 –0.15 Hz); HF indicates high-frequency power (0.15–0.4 Hz); CI indicates confidence interval; Q1, Q2, Q3, and Q4 indicate the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} quartile.

*The percent change in HRV per 10 $\mu g/m^3$ increase in $PM_{2.5}$ concentration. Results were adjusted for age; body mass index; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise; household income; the use of calcium channel blocker, β-blocker, and angiotensin-converting enzyme inhibitor; room temperature; outdoor apparent temperature; season; weekday; and visit date.

†To examine effect modification by position-specific *TLR2* methylation, an interaction term between position-specific *TLR2* methylation and $PM_{2.5}$ was included in the model.

Figure 3.4. Effect of dietary flavonoid (N=497) and folic acid (N=482) intake on mean and position-specific *Toll-like Receptor 2* (*TLR2*) methylation



IQR indicates interquartile-range. Results were adjusted for age, body mass index, total caloric intake, total vitamin C intake, total fiber intake, smoking status, household income, and physical activity.

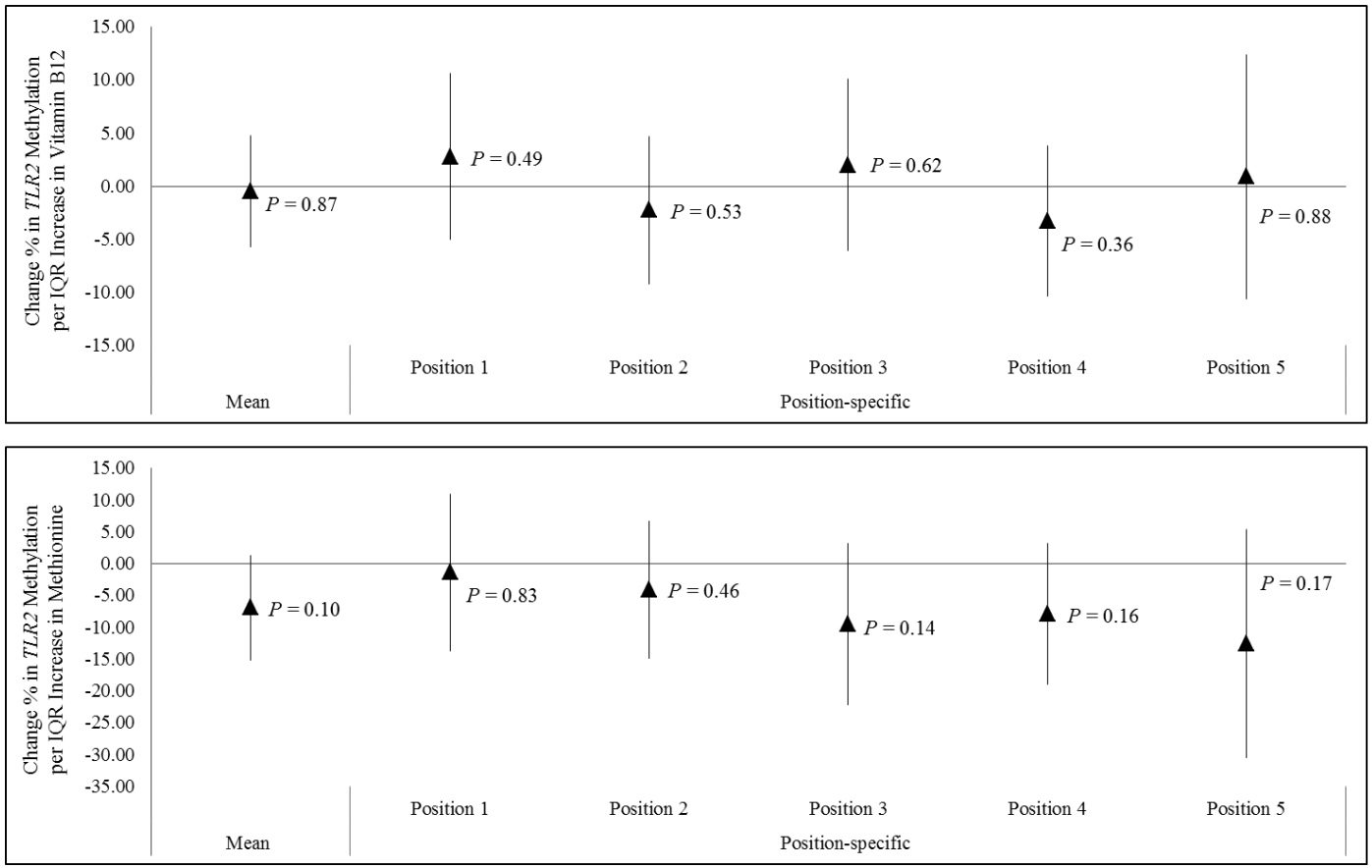
Table 3.6. Effect of dietary flavonoid intake on mean and position-specific *Toll-like Receptor 2* (*TLR2*) methylation, adjusted for total fruit and vegetable intake

	Original model (N=497)*			Adjusted for total fruit and vegetable intake (N=482)†		
	% Change	95% CI	*P*	% Change	95% CI	*P*
Position 1	-10.02	-17.37 to -2.66	<0.01	-8.47	-15.94 to -1.01	0.03
Position 2	-3.81	-10.31 to 2.69	0.25	-2.51	-9.33 to 4.30	0.48
Position 3	-4.49	-12.11 to 3.14	0.25	-4.28	-12.11 to 3.55	0.28
Position 4	-6.05	-12.74 to 0.65	0.08	-6.61	-13.51 to 0.29	0.06
Position 5	-3.31	-14.26 to 7.64	0.55	-4.82	-16.12 to 6.48	0.41
Mean	-5.09	-10.06 to -0.12	0.05	-4.99	-10.16 to 0.17	0.06

*Results were adjusted for age, year of visit, body mass index, total caloric intake, total vitamin C intake, total fiber intake, smoking status, household income, and physical activity.

†In addition to all the covariates in the original model except total vitamin C and fiber intake, results were adjusted for total fruit and vegetable intake.

Figure 3.5. Effect of dietary vitamin B12 (N=497) and methionine (N=482) intake on mean and position-specific *Toll-like Receptor 2* (*TLR2*) methylation



IQR indicates interquartile-range. Results were adjusted for age, body mass index, total caloric intake, total vitamin C intake, total fiber intake, smoking status, household income, and physical activity.

Table 3.7. The effect of particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) exposure on heart rate variability (HRV) at different daily flavonoid intake level, Normative Aging Study, 2000-2011 (N=513)

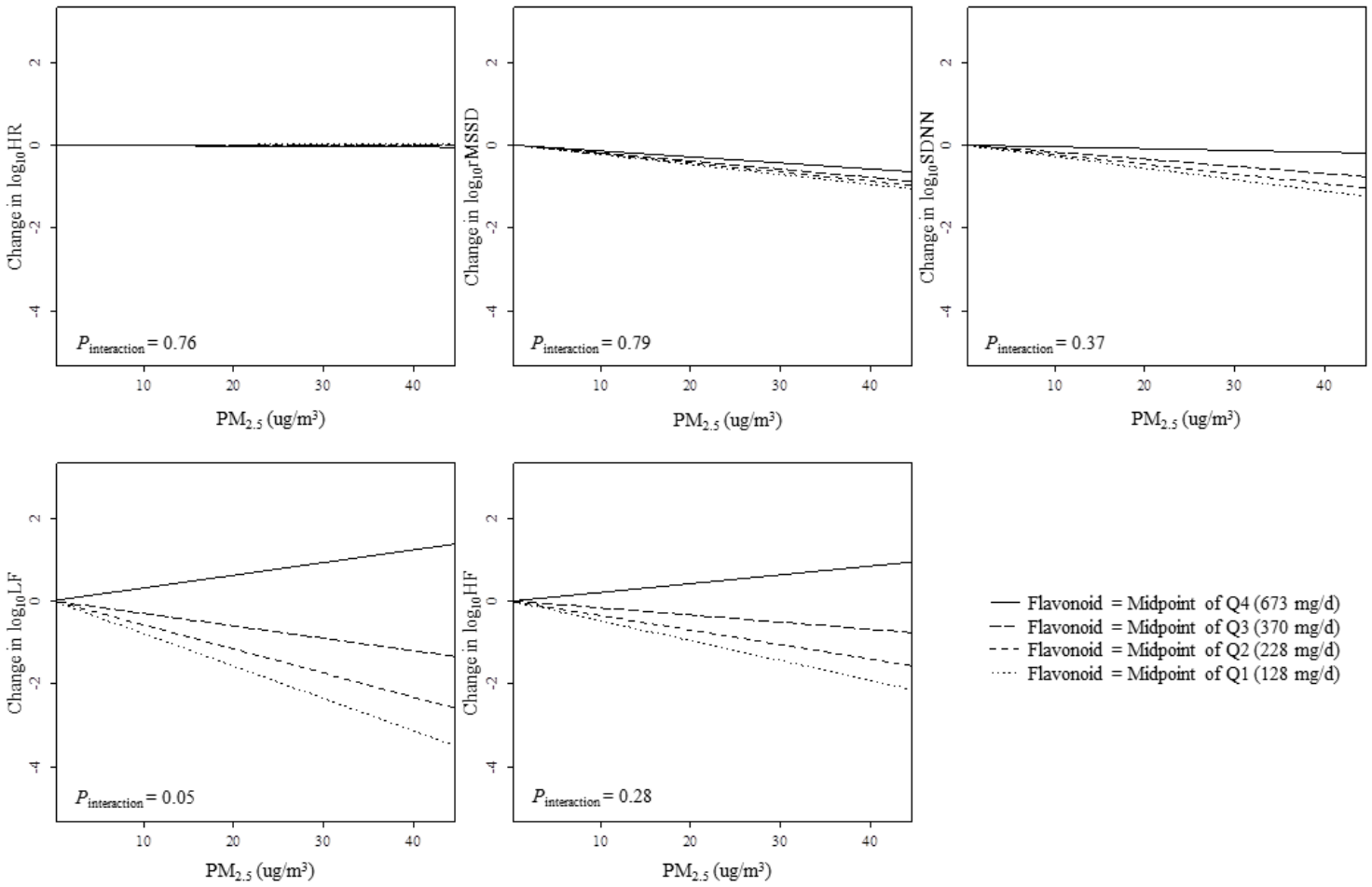
	HR		
Flavonoid Intake	% Change*	95% CI	*P*
Midpoint of Q1 (128 mg/d)	0.06	-1.65 to 1.80	0.95
Midpoint of Q2 (228 mg/d)	0.00	-1.51 to 1.53	1.00
Midpoint of Q3 (370 mg/d)	-0.09	-1.45 to 1.30	0.90
Midpoint of Q4 (673 mg/d)	-0.26	-1.98 to 1.48	0.76
P for Interaction			0.76
	rMSSD		
Flavonoid Intake	% Change*	95% CI	*P*
Midpoint of Q1 (128 mg/d)	-5.32	-16.91 to 7.88	0.41
Midpoint of Q2 (228 mg/d)	-4.94	-15.25 to 6.62	0.39
Midpoint of Q3 (370 mg/d)	-4.41	-13.82 to 6.03	0.39
Midpoint of Q4 (673 mg/d)	-3.25	-15.28 to 10.49	0.63
P for Interaction			0.79
	SDNN		
Flavonoid Intake	% Change*	95% CI	*P*
Midpoint of Q1 (128 mg/d)	-6.14	-14.57 to 3.11	0.19
Midpoint of Q2 (228 mg/d)	-5.22	-12.74 to 2.95	0.20
Midpoint of Q3 (370 mg/d)	-3.89	-10.81 to 3.56	0.30
Midpoint of Q4 (673 mg/d)	-1.00	-10.03 to 8.93	0.84
P for Interaction			0.37
	LF		
Flavonoid Intake	% Change*	95% CI	*P*
Midpoint of Q1 (128 mg/d)	-16.48	-31.35 to 1.61	0.07
Midpoint of Q2 (228 mg/d)	-12.55	-26.37 to 3.87	0.13
Midpoint of Q3 (370 mg/d)	-6.65	-20.07 to 9.03	0.39
Midpoint of Q4 (673 mg/d)	7.31	-12.16 to 31.11	0.49
P for Interaction			0.05
	HF		
Flavonoid Intake	% Change*	95% CI	*P*
Midpoint of Q1 (128 mg/d)	-10.42	-28.69 to 12.54	0.34
Midpoint of Q2 (228 mg/d)	-7.78	-24.49 to 12.63	0.43
Midpoint of Q3 (370 mg/d)	-3.89	-19.74 to 15.09	0.67
Midpoint of Q4 (673 mg/d)	4.96	-16.93 to 32.63	0.68
P for Interaction			0.28

HR indicates heart rate; rMSSD indicates the root mean square of successive differences; SDNN indicates the standard deviation of normal-to-normal intervals; LF indicates low-frequency power (0.04 –0.15 Hz); HF indicates high-frequency power (0.15–0.4 Hz); CI indicates confidence interval; Q1, Q2, Q3, and Q4 indicate the 1st, 2nd, 3rd, and 4th quartile.

Results were adjusted for age; body mass index; total caloric intake; total vitamin C intake; total fiber intake; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise; household income; the use of calcium channel blocker, β-blocker, and angiotensin-converting enzyme inhibitor; room temperature; outdoor apparent temperature; season; weekday; and visit date.

*The percent change in HRV per 10 $\mu g/m^3$ increase in $PM_{2.5}$ concentration.

Figure 3.6. The effect of particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) exposure on heart rate variability (HRV) at different flavonoid intake levels, Normative Aging Study, 2000-2011 (N=513)



log_{10}HR indicates log_{10}-transformed heart rate; log_{10}rMSSD indicates log_{10}-transformed root mean square of the successive differences; log_{10}SDNN indicates log_{10}-transformed standard deviation of normal-to-normal intervals; log_{10}LF indicates log_{10}-transformed low-frequency power (0.04 –0.15 Hz); log_{10}HF indicates log_{10}-transformed high-frequency power (0.15–0.4 Hz); Q1, Q2, Q3, and Q4 indicate the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} quartiles.

The association of $PM_{2.5}$ on rMSSD, SDNN, LF, and HF is modified by flavonoid intake, as indicated by the different slopes. The four lines in each figure represent the relationship between $PM_{2.5}$ and HRV when the flavonoid intake at the midpoints of each quartile. If there was no effect modification, the four lines would be the same. Results were adjusted for age; body mass index; total caloric intake; total vitamin C intake; total fiber intake; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise; household income; the use of calcium channel blocker, β-blocker, and angiotensin-converting enzyme inhibitor; room temperature; outdoor apparent temperature; season; weekday; and visit date .

effect was seen for the 2nd, 3rd, and 4th flavonoid quartile (Table 3.7). The effect modification by flavonoid intake was abrogated when mean *TLR2* methylation and the interaction between mean *TLR2* methylation and $PM_{2.5}$ exposure were fitted in the model (Table 3.8).

Correlation of Plasma Inflammatory Markers with *TLR*2 methylation

Increased *TLR2* methylation level was consistently correlated with increased plasma ICAM-1 level, across five CpG positions ($P<0.0001$, $P<0.0001$, $P=0.0002$, $P=0.0004$, $P=0.0003$, respectively) (Table 9). IL8 and VEGF were also positively correlated with *TLR2* methylation level at position 3($P=0.01$, $P=0.02$, respectively) (Table 3.9).

Sensitivity Analyses

The major sources of dietary flavonoids and folic acid are fruits and vegetables, which contain other nutrients that are likely to affect DNA methylation. Therefore, in sensitivity analysis of models including flavonoids, we adjusted for daily total fruit and vegetable intake—instead of total vitamin C and fiber intake—to examine the robustness of our findings. This adjustment only resulted in minor changes in the effect estimates for the association between flavonoid and *TLR2* methylation, as well as of the effect modification by flavonoid of the effect of $PM_{2.5}$ exposure on HRV (Tables 3.6 and 3.8).

Due to the high tissue and cell-type specificity of DNA methylation, DNA methylation in whole blood might be affected by differences in the proportions of leukocyte cell types.[36] We therefore examined the correlation between the percentage of specific leukocyte types (i.e., eosinophils, lymphocytes, neutrophils, monocytes, and basophils) and *TLR2* methylation level. Among these

five leukocyte types, the percentage of lymphocytes and neutrophils in total blood cells were correlated with *TLR2* methylation (Table 3.10). Therefore, we performed sensitivity analysis adjusted for lymphocyte and neutrophil percentages. Our findings were not affected by this adjustment (Table 3.11).

Table 3.8. Effect modification by flavonoid intake on the association between particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) exposure and heart rate variability (HRV), adjusted for total fruit and vegetable intake, and adjusted for mean *Toll-like Receptor 2* (*TLR2*) methylation

	Original model (N=513)*			Adjusted for total fruit and vegetable intake (N=482)†			Adjusted for mean *TLR2* methylation (N=423)‡		
HRV measure	% Change§	95% CI	*P* for Interaction	% Change§	95% CI	*P* for Interaction	% Change§	95% CI	*P* for Interaction
HR	-0.18	-1.32 to 0.98	0.76	-0.30	-1.48 to 0.90	0.62	0.89	-0.72 to 2.52	0.30
rMSSD	1.17	-7.38 to 10.51	0.79	0.67	-8.45 to 10.7	0.89	0.07	-11.28 to 12.87	0.99
SDNN	2.98	-3.43 to 9.81	0.37	2.77	-3.88 to 9.88	0.42	2.28	-6.49 to 11.87	0.62
LF	14.63	0.27 to 31.03	0.05	15.81	0.50 to 33.45	0.04	10.48	-8.39 to 33.24	0.30
HF	8.98	-6.93 to 27.62	0.28	7.65	-9.17 to 27.59	0.40	7.65	-12.28 to 32.10	0.48

HR indicates heart rate; rMSSD indicates the root mean square of successive differences; SDNN indicates the standard deviation of normal-to-normal intervals; LF indicates low-frequency power (0.04 –0.15 Hz); HF indicates high-frequency power (0.15–0.4 Hz); CI indicates confidence interval.

*Results were adjusted for age; body mass index; total caloric intake; total vitamin C intake; total fiber intake; fasting glucose level; hypertension; smoking status; alcohol consumption; physical exercise; household income; the use of calcium channel blocker, β-blocker, and angiotensin-converting enzyme inhibitor; room temperature; outdoor apparent temperature; season; weekday, and visit date.

†In addition to all the covariates in the original model except total vitamin C and fiber intake, results were adjusted for total fruit and vegetable intake.

‡In addition to all the covariates in the original model, results were adjusted for mean *TLR2* methylation, and the interaction between mean *TLR2* methylation and $PM_{2.5}$ concentration.

§The additional percent change in HRV associated with 10 $\mu g/m^3$ $PM_{2.5}$ concentration, compared to participants with one interquartile range lower flavonoid intake level.

Table 3.9. Correlation between mean blood *Toll-like Receptor 2* (*TLR2*) methylation and plasma inflammatory markers

	TLR2 methylation, Position 1		*TLR2* methylation, Position 2		*TLR2* methylation, Position 3		*TLR2* methylation, Position 4		*TLR2* methylation, Position 5	
	Correlation Coefficient	*P**	Correlation Coefficient	*P**	Correlation Coefficient	*P**	Correlation Coefficient	*P**	Correlation Coefficient	*P**
IL6	-0.067	0.08	-0.025	0.52	0.067	0.08	0.040	0.30	0.020	0.60
IL8	-0.004	0.93	0.006	0.88	0.095	0.01	0.053	0.17	0.049	0.20
IL1β	-0.031	0.42	0.040	0.31	0.046	0.23	0.019	0.63	0.015	0.70
TNFα	-0.043	0.27	-0.056	0.15	0.006	0.87	-0.009	0.81	-0.039	0.32
TNFγ	-0.030	0.44	-0.007	0.86	-0.017	0.66	-0.029	0.45	-0.022	0.58
CRP	0.032	0.35	-0.007	0.85	0.011	0.76	0.024	0.49	0.047	0.17
ICAM-1	0.138	<0.0001	0.159	<0.0001	0.127	0.0002	0.123	0.0004	0.124	0.0003
VEGF	-0.022	0.57	-0.016	0.68	0.091	0.02	0.050	0.20	0.046	0.24

IL indicates interleukin; TNF indicates tumor necrosis factor; CRP indicates C-reactive protein; ICAM-1 intercellular adhesion molecule 1; VEGF indicates vascular endothelial growth factor.

**P* value represents Prob > |r| under H_0: Rho (Spearman's rank correlation coefficient) = 0

Table 3.10. Correlation between mean blood *Toll-like Receptor 2* (*TLR2*) methylation level and proportions of different leukocyte cell types

	Spearman correlation with mean *TLR2* methylation*	
Cell Type	Correlation Coefficient	*P*†
Eosinophils (%)	0.051	0.145
Lymphocytes (%)	0.121	0.001
Neutrophils (%)	-0.118	0.001
Monocytes (%)	0.018	0.604
Basophils (%)	0.012	0.730

*Mean *TLR2* methylation level across five CpG sites.

†*P* value represents Prob > |r| under H_0: Rho (Spearman's rank correlation coefficient) = 0

Table 3.11. Effect modification by position-specific *Toll-like Receptor 2* (*TLR2*) methylation level, on the association between particulate matter with aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) exposure and heart rate variability (HRV), adjusted for cell types

Position	HR	
	% Change[§], Original model, N=500*	% Change[§], Adjusted for cell types, N=498†
1	0.05	0.03
2	0.17	0.26
3	0.53	0.60
4	0.36	0.44
5	0.83‡	0.85‡
Position	rMSSD	
	% Change[§], Original model, N=500*	% Change[§], Adjusted for cell types, N=498†
1	-10.07‡	-9.64‡
2	-5.60‡	-6.10‡
3	-7.35‡	-7.45‡
4	-6.55‡	-6.48‡
5	-8.29‡	-7.88‡
Position	SDNN	
	% Change[§], Original model, N=500*	% Change[§], Adjusted for cell types, N=498†
1	-5.34‡	-5.01‡
2	-0.34	-0.72
3	-3.87‡	-3.97‡
4	-1.62	-1.56
5	-4.58‡	-4.21‡
Position	LF	
	% Change[§], Original model, N=500*	% Change[§], Adjusted for cell types, N=498†
1	-2.45	-1.65
2	1.75	1.39
3	-8.77‡	-8.90‡
4	-1.22	-1.23
5	-7.09	-6.46
Position	HF	
	% Change[§], Original model, N=500*	% Change[§], Adjusted for cell types, N=498†
1	-9.72	-8.72
2	-5.31	-6.35
3	-11.01‡	-11.31‡
4	-7.63	-7.74
5	-14.20‡	-13.33‡

HR indicates heart rate; rMSSD indicates the root mean square of successive differences; SDNN indicates the standard deviation of normal-to-normal intervals; LF indicates low-frequency power (0.04 –0.15 Hz); HF indicates high-frequency power (0.15–0.4 Hz).

*Results were adjusted for age, body mass index, fasting glucose level, hypertension, smoking status, alcohol consumption, physical exercise, household income, medication use (β-blocker, calcium channel blocker, ace inhibitor), room temperature, outdoor apparent temperature, season, weekday, and visit date.

†Results were adjusted for all covariates in the original model and the percentage of neutrophil and lymphocyte.

‡$P \leq 0.05$, statistically significant

§The additional percent change in HRV associated with 10 μg/m^3 increase in $PM_{2.5}$ concentration, compared to participants with one %5mC lower *TLR2* methylation level.

DISCUSSION AND CONCLUSION

This study on a cohort of aging male Boston-area residents demonstrated the adverse impact of short-term $PM_{2.5}$ exposure estimated at the home address on HRV. In addition, novel findings showed that individuals with higher methylation in the *TLR*2 gene promoter are more susceptible to reduced HRV after $PM_{2.5}$ exposure, compared to those with lower methylation levels. Further, higher flavonoid intake in the year preceding the examination significantly lowered *TLR*2 methylation, and attenuated the association between $PM_{2.5}$ exposure and the LF measure of HRV.

PM exposure is a modifiable factor that contributes to cardiovascular morbidity and mortality, particularly in the acute exposure period, as emphasized by the recent AHA statement on air pollution.[2] Consistent with our previous study,[30] we observed the strongest effect on HRV from $PM_{2.5}$ among major types of air pollutants. The negative association between short-term $PM_{2.5}$ exposure and HRV has been repeatedly observed in general population samples,[37] cardiac and hypertensive patients,[38] asthmatic adults,[39] young adults,[40] and older adults,[4,30] as well as in a recent meta-analysis of 29 different studies (N=18,667).[41] The present study showed effects of $PM_{2.5}$ exposure across four different indices of HRV, but not on HR. This finding suggests that short-term $PM_{2.5}$ exposure may affect the cardiac inter-beat intervals before any appreciable influence is detected on heart rate. SDNN, a marker of the cyclic components, represents the total variability over the seven minutes of ECG monitoring. LF variability is linked to the activity of both sympathetic and parasympathetic nervous system, whereas HF and rMSSD are sensitive to high-frequency heart period fluctuations and reflect the parasympathetic nervous activity. The HRV reductions associated with $PM_{2.5}$ in these four indices may reflect pathophysiological changes in cardiac autonomic balance following exposure, which could be a

potential mechanism linking air pollution and cardiac morbidity and mortality in the aging population.[5]

Many epidemiological studies[4,30] have presented a wide inter-individual variability in responses to short-term air pollution, possibly due to biological characteristics.[7] For example, preexisting cardiac conditions, diabetes, and polymorphism of genes involved in endothelial function may confer higher susceptibility to autonomic dysfunction induced by $PM_{2.5}$ exposure.[4, 30] However, these characteristics are either non-modifiable or already well-recognized conditions with specific treatment guidelines. This study revealed that increased *TLR2* methylation is linked with intensified inflammatory responses, as reflected in elevated plasma IL8, ICAM-1, and VEGF level. In addition, we demonstrated that low *TLR2* methylation, an epigenetic mechanism ensuring immunoregulation, reduces the adverse effects of $PM_{2.5}$ exposure on HRV. *TLR2* provides a critical line of protection by inducing Tregs to limit immune-mediated damage.[13,14] Netea et al. reported a 50% decrease in the number of CD4+CD25+ Treg cells accompanied by an impaired release of immunosuppressive cytokines in *TLR2* null mice compared with wild-type mice, demonstrating the role of TLR2 in immunoregulation.[14] These findings support our hypothesis that high *TLR2* methylation, an epigenetic process associated with suppressed *TLR2* expression, conveys susceptibility to PM exposure-induced HRV reductions by decreasing immunoregulation.

Our data presented notable effect heterogeneity across the five CpG positions within *TLR2* promoter region. The distance from position 1 to position 5 is less than 20 base pairs, thus these sites are likely to share most of the same functional complexes and traits, i.e. CpG islands,

DNaseI hypersensitivity, nuclease accessibility and histone modifications (UCSC genome browser) (Figure 2). However, differential methylation patterns across five positions have been observed in the ENCODE project, as identified by the Illumina Infinium Human Methylation 450 Bead Array platform. This suggests that variable methylation patterns exist at these sites even though the CpGs share common functional complexes.

TLR2 methylation is sensitive to dietary and other behavioral factors.[42] In particular, flavonoids, a major subtype of polyphenols, are established inhibitors for methylation-induced gene silencing.[17,18] Flavonoids inhibit the activity of DNA methyltransferase in a dose-response manner, reverse DNA methylation and can reactivate silenced genes.[17,18,43] Our study showed that dietary flavonoid intake over the previous year is negatively associated with *TLR*2 methylation, indicating that adequate flavonoid intake may be essential to maintain a low, protective *TLR*2 methylation level. In addition, we showed that increased flavonoid intake alleviates the adverse effect of $PM_{2.5}$ exposure on LF, and the effect modification is diminished when the model is adjusted for *TLR*2 methylation. This finding suggests that, at least in part, the average daily flavonoid intake over the preceding year affects individual susceptibility to post-exposure HRV reduction by modulating *TLR*2 methylation level. However, formal mediation analysis was not performed in the present study due to the lack of appropriate statistical methodology to identify the potential pathway between two effect modifiers in repeated measure data.

We also investigated a group of methyl nutrients including folic acid, vitamin B12, and methionine, which are essential component in the one-carbon metabolism pathway that is

expected to favor higher blood DNA methylation.[19] However, no evidence to date demonstrates whether methyl nutrients can specifically modulate immune genes.[44] Our analysis did not show significant correlation between methyl nutrients intakes and *TLR*2 methylation status. In our previously published study, $PM_{2.5}$ was negatively associated with heart rate variability in subjects with lower vitamin B12, and methionine intakes, but not in the higher intake groups. Our findings indicated that the effect modification reported in our previous study by vitamin B12 and methionine intake is likely to act through a different mechanism, independent of *TLR2* methylation.

This study has several strengths, including its prospective study design and relatively large sample size that enabled us to determine effect modifications by *TLR2* methylation and flavonoid intakes. To demonstrate the power of the present study, we performed simulation-based power calculation using linear mixed effect models with compound symmetry covariance structure, accounting for covariates adjustments. Specifically, we generated 1000 simulated datasets, each with N= 500 independent subjects, in which each subject had two (correlated) observations. The exposure, effect modifier and outcome were assumed to be normally distributed. A random intercept linear mixed effect model (with compound symmetry correlation structure) was then fitted to each of the simulated datasets. Using effect sizes for both main effect of $PM_{2.5}$ exposure and the effect modification after adjustment for covariates estimated from our data, we expected to have >90% power to detect the observed main effect of PM on HRV indices, and >80% power to detect the observed effect modification by *TLR2* methylation and flavonoid intakes at alpha=0.05 level. Exposure misclassification, which is inherent in epidemiological studies on air pollution, is a limitation of the present study. Most previous studies relied on a few monitoring

sites in the study area as surrogates for recent $PM_{2.5}$ exposure history; that approach introduced measurement errors leading to underestimation of the effect.[45] We reduced misclassification by using a novel, validated, hybrid prediction model to assess temporally and spatially resolved $PM_{2.5}$ exposure level,[24] and the observed effect size of $PM_{2.5}$ exposure on HRV is larger compared to previous study from the same cohort using monitoring site data.[4] This prediction model accounted for the spatial variability of $PM_{2.5}$ concentration within the study area and produced more accurate estimates of recent $PM_{2.5}$ exposure at a 10 x 10 km resolution. The Boston metropolitan area comprises 15 corresponding model grid cells, and the use of the cell-specific predictions for each participant enabled us to assign different exposure levels to participants on a relatively small grid based on their residential areas.[26] Although measurement error cannot be completely avoided, it was unlikely to be associated with participants' HRV status. Therefore, misclassification is likely non-differential and is expected to bias our result towards the null. Acknowledging that confounding is a critical concern in any observational study; we included in regression models an extensive list of covariates. We conducted further analysis to evaluate the sensitivity of our results to covariate specification, and our results were stable and robust. We also examined the correlation between 48-hour and 1-year moving average of $PM_{2.5}$ to rule out the possibility that the observed effect was partially due to correlation of the 48-hour moving average with long-term exposure to $PM_{2.5.}$ These two exposure metrics were not correlated ($R^2 = 0.07$); therefore the association between the 48-hour moving average of $PM_{2.5}$ and HRV was unlikely to be confounded by long-term $PM_{2.5}$ exposure. In addition, all HRV measures were conducted at the same time of the day to eliminate confounding due to diurnal variation. While residual confounding due to unmeasured variables is not unlikely, chances that

the observed association and effect modification reflected bias resulting from residual confounding are minimized.

We acknowledge several other limitations in the present study. During the follow-up of the present study – when data on PM exposure is available – there were 636 eligible NAS participants (Figure 1). However, our analysis only included a study population of 573 participants due to missing PM exposure or HRV data. To examine if the final analysis sample is different from the eligible population, we compared key baseline characteristics (age, race, physical activity, alcohol consumption, smoking status, hypertension, diabetes, and household annual income) between participants excluded in the final analysis due to missing exposure or outcome (N=63) and the participants for final analysis (N=573) and no significant difference was observed (data not shown). In addition, in the analysis investigating effect modification by *TLR2* methylation, 73 participants out of 573 were excluded due to lack of DNA samples for methylation analysis. In the analysis of flavonoid intake and *TLR2* methylation, 60 participants out of 573 were excluded due to lack of FFQ data. However, based on the study operations, it is reasonable to assume that the missingness was independent of participants' exposure level, HRV status, and methylation states. We compared the HRV measures between those with complete data (N=500/N=513) and those without (N=73/N=60), and no apparent difference was observed (data not shown). Therefore, selection bias due to informative missingness in this study was unlikely. Both the within-person and between-person effects contributed to the effect modification by *TLR2* methylation. However, during the study period, the effect modification by *TLR2* methylation was mostly due to between-person effects, as when the models were adjusted for subject id, the significance of effect modification was diminished (data not shown). Due to

the limited number of within-subject repeated visits, we were unable to identify the exact magnitude of effect modification by *TLR2* methylation within subject. In addition, our findings are limited to male older individuals who were residing in a lightly-polluted urban area. Therefore, the conclusion might not be generalizable to young adults, females, or people living in other areas due to differential environmental and physiological factors.

Finally, alveolar inflammation and the subsequent systemic inflammation are essential, but not the only mechanism linking $PM_{2.5}$ exposure and cardiac dysfunction. For example, particles may perturb the autonomic nervous system balance via interaction with lung receptors; a small fraction of PM (ultrafine particles, and some PM constituents such as organic compounds and metals) may translocate into the systemic circulation.[2] Due to the less than comprehensive analysis on plasma inflammatory marker profile and limited sample size, we were unable to draw definite conclusion on the mechanistic pathway underlying the protective effect of low *TLR2* methylation status and high flavonoid intake. Future studies are warranted to identify the precise pathophysiological processes of PM-induced cardiovascular responses, as well as the mechanistic underpinnings of the effect of *TLR2* methylation and dietary flavonoid intake.

The U.S. is an aging society and older people are especially susceptible to air pollution-triggered cardiovascular disease. As air pollution remains an important ubiquitous public health threat, $PM_{2.5}$-associated HRV reduction is an alarming signal of influences on the autonomic regulation of heart rate in older people. These alterations may represent a primary sentinel effect to identify the influence of $PM_{2.5}$ among older individuals or even reflect intermediate mechanisms that may favor acute cardiovascular events.[2,3] Providing preventive strategies at the individual level is

especially important in cardiovascular research, which was recognized as an important new emphasis in the AHA statement on air pollution.[2] Our study provides a novel perspective on individual susceptibility to adverse cardiovascular effects following short-term air pollution; our findings suggest high *TLR2* methylation as a modifiable "epigenetic predisposition" for cardiovascular responses that are associated with short-term $PM_{2.5}$ exposure in older people. In addition, based on our data, increasing flavonoid intake—which could be achieved by increasing intake of one or more of the major sources of dietary flavonoids (tea, beans, wine, berries, and citrus fruits)—may help maintain a low *TLR2* methylation level and mitigate $PM_{2.5}$ exposure-induced cardiovascular system impairment.

In summary, our findings suggest that the epigenetic regulation of TLR2-related immunity may determine vulnerability of older individuals when confronted with air pollution peaks, and that adequate dietary flavonoid intake may ensure a protective epigenetic status against the adverse effects of $PM_{2.5}$ exposure.

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Conclusion

Ambient $PM_{2.5}$ pollution is a major public health challenge facing the world today.[1] The unclear mechanistic underpinning of $PM_{2.5}$'s health effects remains the major gap in current knowledge – therefore creating enormous challenges to develop preventative strategies. In Chapter one and two, we conducted the first human intervention trial, investigating novel mechanistic pathway linking air pollution and adverse health effects, and potential targeted preventive approaches. Our study for the first time provide experimental evidence showing that ambient $PM_{2.5}$ exposure peak has unfavorable effect on cardiac autonomic function, the immune system, and the epigenome – which, can be counteracted by B vitamins supplementation. Our findings suggest promising opportunities to aid the development of novel pharmaceutical intervention strategies – which is particularly important to reduce the burden of pathologies related to ubiquitous exposures such as $PM_{2.5}$ pollution.

The U.S. is an aging society and older people are especially susceptible to air pollution-triggered cardiovascular disease. In chapter three, we showed that $PM_{2.5}$ induced HRV reduction, an alarming signal of influences on the autonomic regulation of heart rate in older people. These alterations may represent a primary sentinel effect to identify the influence of $PM_{2.5}$ among older individuals or even reflect intermediate mechanisms that may favor acute cardiovascular events. Furthermore, our findings suggest that the epigenetic regulation of TLR2-related immunity may determine vulnerability of older individuals when confronted with air pollution peaks, and that increasing flavonoid intake—which could be achieved by increasing intake of one or more of the major sources of dietary flavonoids (tea, beans, wine, berries, and citrus fruits)—may help maintain a low *TLR2* methylation level and mitigate $PM_{2.5}$ exposure-induced cardiovascular system impairment.